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(54) Title: METHODS TO CONTROL THE HOST RANGE OF RETROVIRAL VECTORS

(57) Abstract: A method for generating and selecting for retroviral vectors having an altered host range, for example an expanded or reduced host range, is disclosed. The method includes manipulating host range control element(s) in a retroviral vector. Methods of using the retroviral vectors are also disclosed.

METHODS TO CONTROL THE HOST RANGE OF RETROVIRAL VECTORS

PRIORITY CLAIM

This application claims priority from U.S. Provisional Application No: 60/257,821, filed December 22, 2000, herein incorporated by reference.

FIELD

The present disclosure relates to methods of generating and identifying retroviral vectors having an altered host range, and methods of their use.

BACKGROUND

In retroviruses, spliced and unspliced retroviral RNAs must be efficiently transported from the nucleus to the cytoplasm. The RNAs of some simple retroviruses, such as the Mason-Pfizer Monkey Virus (MPMV) contain a specialized structure called a Constitutive Transport Element (CTE). The CTE is recognized by cellular factors and aids in nuclear export of unspliced viral RNAs. This process is necessary for the expression of viral proteins, the packaging of viral genomic RNA, and the release of replication competent viruses.

CTEs of avian and mammalian retroviruses have been described. Rizvi *et al.* (*Virology* 244:517-32, 1996) disclose that the CTE of MPMV is a *cis*-acting element critical for the replication of MPMV. The CTE facilitates the transport of viral mRNA from the nucleus in a similar manner to the Rev/RRE system of HIV and SIV. Without the CTE there was a six-fold increase in MPMV RNA sequestered in the nucleus compared with the cytoplasm.

Bray *et al.* (*Proc. Natl. Acad. Sci. USA* 91:1256-60, 1994) teach a 219 nucleotide element in the 3' untranslated region of the genome of MPMV (located between *env* and the 3' LTR) that can functionally substitute for the Rev protein and its RRE sequence in promoting transport of unspliced HIV RNAs. Nasioulas *et al.* (*Proc. Natl. Acad. Sci. USA* 92:11940-4, 1995), teach that the HIV 1 Rev/RRE system can increase the expression of Avian Leukosis Virus (ALV) structural proteins in mammalian cells and promote the release of mature ALV from these cells, demonstrating that ALV viral replication is dependent on appropriate post-transcriptional RNA regulation. Ogert *et al.* (*J. Virol*, 70:3834-43, 1996) teach an avian retroviral RNA element that promotes unspliced RNA accumulation in the cytoplasm and thereby promotes Rev-independent expression of HIV protein.

In U.S. Patent No. 5,585,263 a *cis*-acting retroviral mRNA "constitutive enhancer element" is described that promotes transport of intron-containing mRNA and, in conjunction with certain proteins, provides for *rev*-independent expression of certain HIV genes.

Researchers have extended the host range of retroviral vectors by manipulating envelope proteins. Although the host range was extended such that the virus could infect different cell types, the viruses were not replication competent in these cells. U.S. Patent No. 5,591,624 and U.S. Patent

No. 5,716,832, teach the production of recombinant retroviruses adapted to infect a particular cell type, such as a tumor, by manipulating the binding specificity of those retroviruses. U.S. Patent No. 5,512,421 discloses manipulation of the host-range of retroviral vectors by alteration of the retroviral envelope protein so that the vector may infect a wide-range of non-mammalian cells. Barsov and Hughes (*J. Virol.* 70:3922-9, 1996) disclose the manipulation of host-range of a Rous Sarcoma Virus (RSV) derived vector by the substitution of the avian *env* gene with a mammalian *env* gene.

Heterologous genes have been expressed in retroviral vectors. U.S. Patent No. 5,252,465 teaches the expression of heterologous genes in avian erythroblastosis virus vectors. U.S. Patent No. 5,652,130 discloses the use of retroviral vectors for expressing Tumor Necrosis Factor (TNF). U.S. Patent No. 5,635,399 teaches retroviral vectors expressing cytokine genes in retroviral vectors. Barsov and Hughes (*J. Virol.* 70:3922-9, 1996) teach the expression of MLV *env* in a Rous Sarcoma Virus derived vector.

Retroviral vectors are widely used in laboratory research and can be used for clinical gene therapy. Currently used retroviral vectors, however, have certain inherent disadvantages. In some cases, mammalian retroviral vectors can recombine with other endogenous mammalian viruses, which poses obvious clinical dangers (and regulatory approval problems) in gene therapy. Also, presently used retroviruses commonly require a "helper" virus for successful infection and replication, making them difficult to use.

SUMMARY OF THE DISCLOSURE

There is a need for a retroviral vector that is easily manipulated with respect to host range. There is also a need for a retroviral vector that reduces the probability of recombination with endogenous viruses of a subject, such as mammalian viruses, and that needs no "helper" virus or special cell line. There is also a need for a method by which a user may manipulate the host range of a selected vector. Such a retroviral vector would be more useful, easier to use, and more flexible than current retroviral vectors, both in the laboratory and in clinical applications.

The host range of a retrovirus can be expanded in at least two different ways. In the first, a change in the envelope alters the types of cells or organisms that can be infected by the virus. However, this type of manipulation does not necessarily change the cells in which the virus can replicate. Viruses can infect cell in which the virus does not replicate. In such cases the infected cell does not produce infectious virons. Alternatively, viruses can be modified to alter the range of cells or organisms that produce infectious virions. This disclosure concerns the second type of modification.

The present disclosure teaches a method for generating and selecting for retroviral vectors and retroviral particles having an altered host range. The present disclosure also provides the retroviral vectors obtained using the disclosed methods as well as methods of their use.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing how RCASBP(A)ΔDR containing MLV ampho inserts was generated.

FIG. 2 shows the nucleotide sequence of a 196-bp MLV ampho insert in the context of the MLV U3.

FIG. 3 shows the nucleotide sequence of the U3 insert following growth on both DF-1 and 293R(A) cells.

FIG. 4 is the nucleotide sequence of the portion of the *gag* gene in RCAS vectors containing point mutations identified in a DR-deleted ALV containing an MLV insert. The normal splice donor (sd) and the cryptic splice donor (cryptic sd) sites are shown in bold face type. The first six residues that were mutated are numbered in bold face type, with the nucleotides present following mutation below the wild-type sequence.

FIG. 5A and 5B are schematic diagrams of modified RCASBP vectors (A) RCASBPΔDRNg1-9 and (B) RCASBPΔDRNg1-9gfp. The SA shown is used to generate the spliced message containing gfp.

FIG. 6 is the nucleotide sequence of the portion of the *gag* gene in RCAS vectors containing point mutations identified in a DR-deleted ALV containing an MLV insert, showing the additional two mutations (7 and 8) acquired by long-adapted virus. The second cryptic splice donor site is shown in bold face type.

FIG. 7 is a digital image of a Southern blot, showing that many proviruses present in 293R(A) cells infected with viral supernatants produced on 293R(A) cell were extensively deleted: Lane 1. RCASBPΔDRNg1-9gfp plasmid DNA digested with EcoRI (5 ng); Lane 2. Genomic DNA from uninfected 293R(A) cells digested with EcoRI. Lane 3. Hirt DNA isolated from DF-1 cells infected with long-adapted RCASBPΔDRNg1-9gfp. Lane 4. Genomic DNA from DF-1 cells infected with RCASBPΔDRNg1-9gfp. Lane 5. Genomic DNA from 293R(A) cells infected with long-adapted RCASBPΔDRNg1-9gfp virus produced on DF-1 cells. Lanes 6-9. Genomic DNA from 293R(A) cells infected with long-adapted RCASBPΔDRNg1-9gfp virus produced on 293R(A) cells at different cell passages following infection (P7, P11, P10, P13, respectively). Lane 10. Genomic DNA from 293R(A) cells infected with mid-adaptation RCASBPΔDRNg1-9gfp virus produced on 293R(A) cells. Lane 11. RCASBPΔDRNg1-9 plasmid DNA digested with EcoRI (5 ng).

FIG. 8 is a schematic drawing of the ^{32}P labeled fragments of RCASBP(A) DNA used as probes to determine which portions of the provirus remained in 293R(A) cells, (A) *gag* gene probe generated by digesting RCASBP(A) with PvuII and gel isolating the resulting ~1100-bp fragment; (B) *pol* gene probe generated by digesting RCASBP(A) with AvrII and gel isolating the resulting ~1900-bp fragment; and (C) *env* gen probe generated by digesting RCASBP(A) with KpnI and SalI and gel isolating the resulting ~1000-bp fragment.

FIG. 9 is a schematic drawing showing differential splice donor sites.

FIG. 10 is a schematic diagram showing a summary of the pRR145 deletion mutants.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NO: 1 shows a 96 bp nucleic acid sequence derived from the LTR of MLV, which is located between nucleotides 355 and 450 of SEQ ID NO: 3.

SEQ ID NO: 2 shows a 196 bp *Alu*I fragment nucleic acid sequence derived from the LTR of MLV, which is located between nucleotides 237 and 456 of SEQ ID NO: 3.

SEQ ID NO: 3 shows the sequence of an MLV LTR.

SEQ ID NO: 4 shows an exemplary forward primer used to amplify SEQ ID NO: 1.

SEQ ID NO: 5 shows an exemplary reverse primer used to amplify SEQ ID NO: 1.

SEQ ID NO: 6 shows an exemplary forward primer used to amplify DNA sequences cloned into the viral *Cla*I site for clone selection of viruses containing SEQ ID NO: 1.

SEQ ID NO: 7 shows an exemplary reverse primer used to amplify DNA sequences cloned into the viral *Cla*I site for clone selection of viruses containing SEQ ID NO: 1.

SEQ ID NO: 8 shows an exemplary forward primer used to amplify DNA sequences cloned into the viral *Cla*I site for clone selection of viruses harboring the 196 bp insert.

SEQ ID NO: 9 shows an exemplary reverse primer used to amplify DNA sequences cloned into the viral *Cla*I site for clone selection of viruses harboring the 196 bp insert.

SEQ ID NO: 10 is a nucleic acid sequence of a 38-bp downstream flanking sequence.

SEQ ID NO: 11 is a nucleic acid sequence containing a portion of a *gag* gene with mutations selected for after shot-term adaptation.

SEQ ID NO: 12 is a nucleic acid sequence containing a portion of a wild-type *gag* gene.

SEQ ID NO: 13 is a nucleic acid sequence containing a portion of a *gag* gene with mutations selected for after one year of adaptation.

SEQ ID NOS: 14 and 15 are exemplary nucleic acid sequences of PCR primers that can be used to amplify U3 flanking sequences.

SEQ ID NOS: 16-19 are exemplary nucleic acid sequences of PCR primers that can be used to sequence regions of a *gag* gene.

SEQ ID NOS: 20-21 are exemplary nucleic acid sequences of PCR primers that can be used to identify deletions of proviral sequences.

SEQ ID NOS: 22-23 are exemplary nucleic acid sequences of PCR primers that can be used to detect unspliced RNA in virus particles.

SEQ ID NOS: 24-25 are exemplary nucleic acid sequences of PCR primers that can be used to detect aberrantly spliced RNA in virus particles.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Abbreviations and Terms

The following explanations of the terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. As used herein, "comprising" means" "including" and the singular forms "a" or "an" or "the" include plural references unless the context clearly dictates otherwise. For example, reference to "comprising a cell" includes one or a plurality of such cells, and reference to "comprising the retrovirus" includes reference to one or more retroviruses and equivalents thereof known to those skilled in the art, and so forth.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs.

ALV: Avian leukosis virus

ASLV: Avian sarcoma leukosis virus

GFP: Green fluorescent protein

HIV: Human immunodeficiency virus

MLV: Murine Leukemia virus

MMTV: Mouse Mammary Tumor Virus

MPMV: Mason-Pfizer monkey virus

RCAS: Replication competent ALV LTR with a splice acceptor vector

RCASBP: Replication competent ALV LTR with a splice acceptor vector containing the Bryan polymerase

RSV: Rous Sarcoma Virus

RT: Reverse transcriptase

SIV: Simian immunodeficiency virus

Altered host range: The host range of a retrovirus is altered when the cell(s) in which a retrovirus is replication competent is changed. Host range can be altered by expanding or contracting (reducing) the host range of a retrovirus. In one embodiment, the host range of a retrovirus can be altered by manipulating a host-range control element (HRCE) of a retrovirus using methods provided herein.

In one example, the host range of a retrovirus is altered by expanding the host range of the retrovirus. In one specific non-limiting example, ASLV is replication competent in avian, but not mammalian cells. The host range of ASLV is expanded if it gains the ability to perform at least one round of replication in mammalian cells. ASLV may or may not retain the ability to replicate in avian cells.

In another example, the host range of a retrovirus is altered by contracting or reducing the host range of the retrovirus. In one specific non-limiting example, MLV ampho is replication

competent in both avian and mammalian cells. The host range of MLV ampho is contracted if it looses the ability to replicate in mammalian and/or avian cells.

Amphotrophic virus: A virus that can replicate both in cells of its native host and also in cells of other species. In one specific non-limiting example an amphotrophic virus is MLV ampho which replicates in both mammalian cells (its native host) and in avian cells (its non-native host). In another specific non-limiting embodiment, the amphotrophic virus is SNV (spleen necrosis virus), which replications in both turkey cells (its native host) and dog cells (its non-native host).

Antisense, Sense, and Antigene: Double-stranded DNA (dsDNA) has two strands, a 5' -> 3' strand (the plus strand) and a 3' -> 5' strand (the minus strand). Because RNA polymerase adds nucleic acids in a 5' -> 3' direction, the minus strand of the DNA serves as the template for the RNA during transcription. Thus, the RNA formed will have a sequence complementary to the minus strand, and identical to the plus strand (except that the base uracil is substituted for thymine).

Antisense molecules are molecules that are specifically hybridizable or specifically complementary to either RNA or the plus strand of DNA. Sense molecules are molecules that are specifically hybridizable or specifically complementary to the minus strand of DNA. Antigene molecules are either antisense or sense molecules directed to a DNA target.

Avian: As applied to a virus, refers to any virus that is native to birds. A non-avian virus is any virus that is not native to birds. Avian-derived refers to any cell or virus isolated from a bird, or derived from a cell or virus isolated from a bird.

Binding/stable binding: An oligonucleotide binds or stably binds to a target nucleic acid if a sufficient amount of the oligonucleotide forms base pairs or is hybridized to its target nucleic acid, to permit detection of that binding. Binding can be detected by physical or functional properties of the target:oligonucleotide complex. Binding between a target and an oligonucleotide can be detected by any method known to one skilled in the art, including functional and physical binding assays. Binding can be detected functionally by determining whether binding has an observable effect upon a biosynthetic process such as expression of a gene, DNA replication, transcription and translation.

Physical methods of detecting the binding of complementary strands of DNA or RNA are well known in the art, and include such methods as DNase I or chemical footprinting, gel shift and affinity cleavage assays, Northern blotting, dot blotting and light absorption detection procedures. For example, a method which is widely used, because it is simple and reliable, involves observing a change in light absorption of a solution containing an oligonucleotide (or an analog) and a target nucleic acid at 220 to 300 nm as the temperature is slowly increased. If the oligonucleotide or analog has bound to its target, there is a sudden increase in absorption at a characteristic temperature as the oligonucleotide (or analog) and target dissociate or melt.

The binding between an oligomer and its target nucleic acid is frequently characterized by the temperature (T_m) at which 50% of the oligomer is melted from its target. A higher T_m means a stronger or more stable complex relative to a complex with a lower T_m.

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences which determine transcription. cDNA may be synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

Chimeric retroviral vector: A retroviral vector that includes at least one nucleic acid sequence from a first retrovirus linked to a nucleic acid from a second, non-identical retrovirus. The second sequence can be from a retroviral vector from a different Kingdom, Phylum, class, order, family, genus or species than the first retroviral vector.

Complex ("MC-type") retroviruses: A retrovirus that does not contain CTEs. Specific, non-limiting examples include HIV-1 and SIV. In these viruses, the Rev protein interacts with the Rev-responsive element (RRE), a short *cis*-acting sequence on the viral transcript. The Rev/RRE complex interacts with cellular factors to allow export of mRNA from the nucleus to the cytoplasm where it is expressed.

Constitutive Transport Element (CTE): Also known as a constitutive transport enhancer, a viral element that aids in nuclear export of viral mRNA to the cytoplasm in simple retroviruses. The CTE is typically a *cis*-acting element in the 3' untranslated region of a retrovirus genome between *env* and the 3' LTR. CTEs can complement Rev-REE- mutants of complex viruses (Bray *et al.*, *Proc. Natl. Acad. Sci. USA* 91:1256-60, 1994 and Zolotukhin *et al.*, *J. Virol.* 68:7944-52, 1994).

A non-limiting example of a CTE is the 219 bp *cis*-acting element present in the 3' region of both MPMV and Simian Retrovirus type 1 (SRV-1) (Bray *et al.*, *Proc. Natl. Acad. Sci. USA* 91:1256-60, 1994; Zolotukhin *et al.*, *J. Virol.* 68:7944-52, 1994). See Genbank Accession No. SIVMPCG between nucleotides 8022 and 8240, and discussed in U.S. Patent No. 5,585,263 (column 6). MPMV CTE RNA contains three stem-loop structures, the first of which contains a nine nucleotide motif with 67% homology to the Rev-binding domain of HIV-1 RRE. Another non-limiting example of a CTE is the CTE of RSV, which is found between nucleotides 8770 and 8925 in the RSV genome.

U.S. Patent No. 5,880,276 describes a method for identifying (and making) a CTE (see Example 8), which can be used to identify a CTE for use in the present embodiments. An adaptation of such a method includes:

(1) Isolating a retroviral genomic sequence or cellular genomic sequence having homology to a known CTE (for example, has 50% identity with a known CTE when compared using blastn at default perameters). Such a known CTE could, for example, be a CTE from SRV-1, from MPMV, or from RSV, or could be any CTE characterized by being found in the 3' UTR of a retrovirus genome between *env* and the 3' LTR, that functions to mediate nuclear export of RNA in simple retroviruses;

(2) Insertion of such a sequence into a vector in cis, to produce a vector that is transcribed into mRNA, which is either differentially spliced, alternatively spliced, incompletely spliced or unspliced, and thus, not normally transported from the nucleus to the cytoplasm;

(3) Introduction of such a recombinant vector into non-host cells;

(4) Assaying the cultured cells for expression of the DNA molecule such as by detection of its mRNA in the cytoplasm or production of protein encoded by the DNA molecule, wherein detection of such expression indicates that the sequence comprises a CTE; and

(5) Isolation and purification of the sequence from the recombinant vector using standard methods.

Deletion: The removal of a sequence of DNA, the regions on either side being joined together.

Deoxyribonucleic acid (DNA). A long chain polymer which includes the genetic material of most living organisms (some viruses have genes including ribonucleic acid, RNA). The repeating units in DNA polymers are four different nucleotides, each of which includes one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules code for amino acid in a polypeptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

Derived from: A second virus is derived from a first virus if the second virus genome retains the majority of structural genes of the first, and retains at least 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% sequence identity to the genome of the first virus.

Direct repeat (DR) sequences: Identical or nearly identical sequences of DNA present as two or more copies in the same orientation in the same molecule. Such sequences need not be adjacent.

Envelope polypeptide (Env): An env polypeptide is a retroviral envelope protein which encodes the surface (SU) glycoprotein and the transmembrane (TM) protein of the virion. The SU glycoprotein and the TM protein form a complex that interacts specifically with cellular receptors.

Functional Deletion: A mutation in a sequence that deletes or has an effect equivalent to deletion of the sequence. In one embodiment, the function of a HRCE or an essential gene product is eliminated by a deletion, insertion, or substitution, for example deletion of a portion of the sequence or the entire sequence.

Functionally Equivalent: Nucleic acid sequence alterations in a retroviral vector that yields the same results described herein. Such sequence alterations can include, but are not limited to, conservative substitutions, deletions, mutations, frameshifts, and insertions. In a first retroviral vector with its HRCE functionally deleted, insertion of a sequence from a second retroviral vector, such as an HRCE of the second retroviral vector, which allows the first retroviral vector to be replication competent in the host cell(s) of the first retroviral vector, demonstrates that the second HRCE is functionally equivalent of the first HRCE. However, a functionally equivalent sequence need not confer replication competence in a first retroviral host cell to be complementary, but merely confer replication competence to the first retroviral vector in some cell type. In one embodiment, a segment from the LTR of MLV ampho is functionally equivalent to the DR of ASLV because when DR is

functionally deleted in ASLV and replaced with a sequence from the MLV ampho LTR, the resulting chimeric virus is replication competent in an avian host cell.

Genetic fragment: Refers to any polynucleotide, DNA or RNA, derived from a larger polynucleotide.

Group Specific Antigen Polypeptide or Gag: A gag protein is a retroviral group specific antigen polypeptide which is proteolytically processed into the mature proteins MA (matrix), CA (capsid), and NC (nucleocapsid), and other proteins that are numerically designated. In one embodiment, a portion of a gag protein refers to at least 15 consecutive amino acids of a gag protein sequence. In one embodiment, a portion of an gag protein refers to at least 25 consecutive amino acids of an gag protein sequence. In yet another embodiment, a portion of an gag protein refers to at least 35, for example at least 45, at least 50, at least 100, at least 200, or even at least 300 nucleotides of a gag nucleic acid sequence.

Heterologous: A sequence that is not normally (i.e. in the wild-type sequence) found adjacent to a second sequence. In one embodiment, the sequence is from a different genetic source, such as a virus or organism, than the second sequence.

Host cell: In one embodiment, a host cell is a native cell(s) of a retrovirus which the retrovirus can infect and in which the retrovirus is replication competent. For example, host cells of an avian retrovirus include, but are not limited to any avian cell, such as any chicken, quail, or turkey cell, for example chick embryo fibroblast (CEF) cells and DF-1 cells. In a particular embodiment, the host cell of ASLV includes CEF and DF-1 cells.

In another example, host cells of a mammalian retrovirus include, but are not limited to any mammalian cell, such as any mouse, rat, pig, or human cell, such as any cell from a mammalian subject such as a blood cell, liver cells or lung cell, for example 293 or 3T3 cells.

In yet another example, a host cell includes a cell which is not a native cell of a retrovirus, but which the retrovirus can infect and in which the retrovirus is replication competent. For example, host cells of MLV ampho include mammalian and avian cells, for example 293 and DF-1 cells.

Host range: Refers to the types or species of cells in which a retrovirus or retroviral vector is replication competent. For example, the host range of MLV ampho includes avian and mammalian cell types.

Host range control element (HRCE): A nucleic acid sequence, such as an RNA or DNA sequence of a retrovirus or retroviral vector, that affects the ability of the retrovirus or retroviral vector to be replication competent in a particular type or species of cell. In one embodiment, the HRCE is an RNA sequence of a retrovirus or retroviral vector, but not an env sequence. Examples of HRCEs include, but are not limited to the DR sequence of ASLV; the segment from the LTR of MLV ampho (SEQ ID NO: 3) or portions thereof such as SEQ ID NO: 1 or 2 or 3; gag sequences such as SEQ ID NOS: 11 or 13. In addition to the specific examples of HRCEs described in the present specification, the methods disclosed allow for the identification of HRCEs in any retrovirus or retroviral vector, such as a simple retrovirus. In addition, the methods provided herein allow for the

manipulation of HRCE in a retrovirus or retroviral vector, for example insertion or functional deletions of HRCEs, to control the host range of the retrovirus or retroviral vector.

Infective: A retrovirus or retroviral vector is infective when it transduces a cell, replicates without the benefit of any complementary virus or vector, and spreads progeny vectors or viruses to other cells in an organism or cell culture, where the progeny vectors or viruses have the same ability to reproduce and spread throughout the organism or cell culture. Thus, for example, a nucleic acid encoding a retroviral particle is not infective if the nucleic acid cannot be packaged (e.g. if the retroviral particle lacks a packaging site), even though the nucleic acid can be used to transfect a cell.

Integration: A retrovirus integrates into cellular DNA when a DNA copy of the retroviral genome is incorporated into the cellular genome (i.e. into a chromosome).

Isolated: An "isolated" biological component (such as a nucleic acid or protein has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs.

An isolated nucleic acid is a nucleic acid substantially separated or purified away from other nucleic acid sequences in the cell of the organism in which the nucleic acid naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA. The term isolated encompasses nucleic acids and/or proteins purified by standard purification methods. The term also embraces nucleic acids and/or proteins prepared by recombinant expression in a host cell as well as those chemically synthesized.

Long Terminal Repeat (LTR): A DNA sequence repeated at the 5' and 3' end of an integrated retrovirus genome (the provirus) which is not found in the retroviral RNA genome. LTRs are generated through a replication process prior to integration and consist of three structural regions: U3, R and U5. The LTR generally contains an enhancer sequence(s), promoter sequence(s), 3' RNA processing sequence(s), and integration (att) sequence(s). In a replication competent retrovirus, the LTR may also contain an active RNA polymerase II promoter which allows transcription of the integrated provirus by host cell RNA polymerase II to generate new copies of the retroviral RNA genome. Examples include, but are not limited to the LTR of HIV1 (Patricia *et al.*, *AIDS Res. Hum. Retroviruses* 3:41-55, 1987); MMTV (Lee *et al.*, *Virology* 159:39-48, 1987); MLV, and RSV (Yamamoto *et al.*, *Cell* 22:787-97, 1980).

Mammal: This term includes both human and non-human mammals. Similarly, the terms "patient," "subject," and "individual" include both human and veterinary subjects.

Mammalian: As applied to a virus, refers to any virus native to mammals. Mammalian derived refers to any cell or virus isolated from a mammal.

Marker polypeptide: A polypeptide used to identify cells that express the polypeptide. A marker polypeptide can be detected using methods known to one of skill in the art, including enzymatic assays and assays utilizing antibodies (e.g. ELISA or immunohistochemistry). Specific non-limiting examples of a maker protein are luciferase, green fluorescent protein (GFP), or β-galactosidase.

Native host: A host in which a retrovirus replicates in nature, wherein a non-native host of a retrovirus is not infected in nature.

Non-host cell: A non-host cell includes all cells in which a retrovirus is not native and in which the retrovirus does not replicate. For example, a non-host cell of an avian retrovirus includes, but is not limited to any non-avian cell, such as a mammalian cell. In a particular example, a non-host cell of ASLV includes 293 and 3T3 cells.

Oligonucleotide: A linear polynucleotide sequence of up to about 200 nucleotides in length, for example a polynucleotide (such as DNA or RNA) which is at least 6 nucleotides, for example at least 15, 50, 100 or even 200 nucleotides long.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into a peptide.

Ortholog: Two nucleotide sequences are orthologs of each other if they share a common ancestral sequence and diverged when a species carrying that ancestral sequence split into two species. Orthologous sequences are also homologous sequences.

Packaging Signal: A complex signal, present in viral RNA, also known as "ϕ", that plays a role in the packaging of viral RNA into viral particles.

PCR (polymerase chain reaction): A method in which cycles of denaturation, annealing with primer, and then extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence.

Polymerase (Pol): A pol protein is a retroviral reverse transcriptase, which contains both DNA polymerase and associated RNAse H activities, and Integrase (IN). Pol mediates replication of the viral genome *in vivo*. The ends of the newly synthesized linear double-stranded viral DNA are recognized and two nucleotides from the 3' end of each strand are removed. These DNA ends are joined to a target DNA at random sites.

Probes and primers: A probe includes an isolated nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989); and Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences (1987).

Primers are short nucleic acids, for example DNA oligonucleotides at least 15 nucleotides in length. Primers may be annealed to a complementary target DNA strand by nucleic acid

hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by PCR or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989), Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences (1987), and Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, 1990, Innis *et al.* (eds.), 21-27, Academic Press, Inc., San Diego, California. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Probes and primers disclosed herein comprise at least 15 nucleotides, although a shorter nucleic acid may be used if it specifically hybridizes under stringent conditions with a target nucleic acid by methods well known in the art. The disclosure thus includes isolated nucleic acid molecules that comprise specified lengths of the disclosed sequences. One of skill in the art will appreciate that the specificity of a particular probe or primer increases with its length. Thus, for example, a primer comprising 20 consecutive nucleotides of a gene will anneal to a target sequence contained within a genomic DNA library with a higher specificity than a corresponding primer of only 15 nucleotides. To enhance specificity, longer probes and primers can be used, for example probes and primers that comprise at least 20, 30, 40, 50, 60, 70, 80, 90, 100 or more consecutive nucleotides from any region of the disclosed sequences. By way of example, the sequences disclosed herein may be apportioned into halves or quarters based on sequence length, and the isolated nucleic acid molecules may be derived from the first or second halves of the molecules, or any of the four quarters.

When referring to a probe or primer, the term "specific for (a target sequence)" indicates that the probe or primer hybridizes under stringent conditions substantially only to the target sequence in a given sample comprising the target sequence.

Polynucleotide: A linear nucleic acid sequence of any length. Therefore, a polynucleotide includes molecules which are at least 15, 50, 100, 200 (oligonucleotides) and also nucleotides as long as a full length cDNA.

A **portion** (of a nucleotide sequence) as used herein refers to at least 10, 20, 30, 40, 50, 100 or more contiguous nucleotides of a specified nucleotide sequence, for example the sequences disclosed herein. A portion can include an entire gene or an entire specified sequence, e.g., a portion of a DNA sequence of gene A can include as few as 10 nucleotides, or as many as 50 nucleotides or more, or the entire ORF or the entire gene, so long as the sequence comprises at least 10 nucleotides of the DNA sequence of gene A. For example, a portion of the LTR of MLV ampho can include the entire 614 bp sequence (SEQ ID NO: 3), or as few as 10 nucleotides thereof.

Promoter: An array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

pRR145: An infectious virus generated by replacing a portion of the *pol* and most of the *env* gene of Moloney MLV (MoMLV) with the same sequence from the amphotropic 4070A clone to create an Mo(4070A) chimera designated pRR145 (Ott *et al.*, *J. Virol.* 66:6107-16, 1992). The pRR145 genomic clone contains two complete copies of the viral LTR (see FIG. 10).

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified protein or nucleic acid preparation is one in which the protein or nucleic acid is more pure than the protein or nucleic acid in its natural environment within a cell. For example, a preparation of a protein is purified if the protein represents at least 50%, for example at least 70%, of the total protein content of the preparation.

RCAS Vector: An avian retroviral vector derived from the replication competent ALV with a splice acceptor vector.

Recombinant: A nucleic acid sequence that is not naturally occurring or a sequence made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques, such as those described in Sambrook *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). Such recombinant nucleic acid sequences can be used to produce recombinant proteins.

Replication competent: A virus is replication competent in a particular cell line or cell type if that virus, without the need for a helper virus, can undergo at least one complete replication cycle in the cell by infecting the cell, replicating and assembling in the cell and producing infectious progeny viruses. A virus is replication competent in a non-native cell type if the virus can replicate in a cell from a Kingdom, Phylum, class, order, family, genus or species that is different from the one to which the virus is native.

Replication competency can be assessed using methods disclosed herein, as well as other methods known to those skilled in the art, for example identifying particle production using ELISA or Western blotting using gag antibodies (see also Coffin *et al.*, *Retroviruses*, Cold Spring Harbor Laboratory Press, 1997).

Replication defective: A virus is replication defective if it cannot replicate. In one embodiment, a retroviral vector is replication defective if it cannot replicate in a host cell of the retroviral vector.

Retroviral vector: A nucleic acid sequence which can be packaged into a retrovirus.

Retrovirus: Any virus in the family Retroviridae. These viruses have similar characteristics, specifically they share a replicative strategy. This strategy includes reverse transcription of the virion

RNA into linear double-stranded DNA, and the subsequent integration of this DNA into the genome of the cell. All native retroviruses contain three major coding domains with information for virion proteins: gag, pol and env.

In one example, a retrovirus is a simple retrovirus. In another example, a retrovirus is an avian type C retroviruses, such as avian leukosis virus (ALV). In yet another example, a retrovirus is a BLV-HTLV retrovirus such as bovine leukaemia virus (BLV), a lentivirus such as human immunodeficiency virus (HIV-1), a mammalian type B retrovirus such as mouse mammary tumor virus (MMTV), a mammalian type C retrovirus such as murine leukaemia virus (MLV), a spumavirus such as human spumavirus (HSRV), or a type D retroviruses such as Mason-Pfizer monkey virus (MPMV). In other example, a retrovirus is a Murine leukemia-related virus, an RSV, a human T-cell leukemia virus, a human foamy virus, or an ASLV.

Sample: A material to be analyzed. In one embodiment, a sample is a biological sample. In a specific example, a biological sample contains genomic DNA, cDNA, RNA, or protein obtained from the cells of a subject. Other examples of biological samples, include, but are not limited to: peripheral blood, serum, plasma, urine, cerebrospinal fluid, pleural fluid, synovial fluid, peritoneal fluid, gastric fluid, saliva, lymph fluid, interstitial fluid, sputum, stool, physiological secretions, tears mucus, sweat, milk, semen, seminal fluid, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses, tissue biopsy, surgical specimen, fine needle aspriates, amniocentesis samples and autopsy material.

Sequence identity: The identity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity between the sequences. Sequence identity can be measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more identical the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity when aligned using standard methods. This homology is more significant when orthologous proteins or cDNAs are derived from species which are more closely related (e.g., human and mouse sequences), compared to species more distantly related (e.g., human and C. elegans sequences).

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al. Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894)

and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer than around 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater identity to the reference sequence will show increasing percentage identities when assessed by this method, such as at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% sequence identity, when using gapped blastp with databases such as the nr or swissprot database. Queries searched with the blastn program are filtered with DUST (Hancock and Armstrong, 1994, *Comput. Appl. Biosci.* 10:67-70). Other programs use SEG. When less than the entire sequence is being compared for sequence identity, homologs typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85%, 90%, 95% or 98% depending on their identity to the reference sequence. Methods for determining sequence identity over such short windows are described at the NCBI web site. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that significant homologs can be obtained that fall outside of the ranges provided.

One indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and vary under different environmental parameters. Nucleic acid molecules that hybridize under stringent conditions to a target nucleic acid (or a sequence complementary thereto) typically hybridize to a probe based on either an entire a target nucleic acid (or a sequence complementary thereto) or selected portions of a target nucleic acid (or a sequence complementary thereto), respectively, under wash conditions as described in EXAMPLE 14.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. Changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein. Such homologous peptides may, for example, possess at least 75%, 80%, 90%, 95%, 98%, or 99% sequence identity determined by this method. When less than the entire sequence is being compared for sequence identity, homologs may, for example, possess at least 75%, 85% 90%, 95%, 98% or 99% sequence identity over short windows of 10-20 amino acids. Methods for determining sequence identity over such short windows can be found at the NCBI web site. One of skill in the art will appreciate that the sequence identity ranges are provided for guidance only; it is possible that significant homologs or other variants can be obtained that fall outside the ranges provided.

For comparisons of nucleic acid sequences, sequence identity can be determined by comparing the nucleotide sequences of two nucleic acids using the BLAST sequence analysis software, for instance, the NCBI BLAST 2.0 program gapped *blastn* set to default parameters. (One example of such

default settings would be: expect = 10, filter = default, descriptions = 500 pairwise, alignments = 500, alignment view = standard, gap existence cost = 11, per residue existence = 1, per residue gap cost = 0.85). Nucleic acids with even greater identity to a reference nucleic acid sequence will show increasing percentage identities when assessed by this method, such as at least 50%, 60%, 70%, 75%, 80%, 90%, 95%, 98%, or 99% sequence identity of the nucleotides.

An alternative (and not necessarily cumulative) indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Species: A group of organisms of common ancestry that are able to reproduce only among themselves and that are usually geographically distinct. In one embodiment, mammals and birds are different species. In another embodiment, chickens and quails are different species.

Simple ("S-type") Retrovirus: Simple retroviruses have only three genes, *gag*, *pol* and *env*, which encode viral enzymes and structural proteins. Examples of simple retroviruses include, but are not limited to, ASLV, RSV, SNV, MFMV, and MLV (see Coffin *et al.*, *Retroviruses*, Cold Spring Harbor Laboratory Press, 1997). The cytoplasm of cells infected with most simple retroviruses normally contain only two viral transcripts, the spliced message that is translated into Env proteins and the full length RNA. RSV produces an additional spliced message that allows the expression of the *src* oncogene. Genomes of some simple retroviruses contain *cis*-acting RNA structural elements analogous to the response elements of complex viruses.

Subject: Living multicellular vertebrate organisms, a category which includes, both human and veterinary subjects for example, mammals and birds.

Supernatant: The culture medium in which a cell is grown. The culture medium may include material from the cell. If the cell is infected with a virus, the supernatant can include viral particles.

Target Nucleic Acid: Refers to a nucleic acid, such as ssDNA, dsDNA or RNA, that hybridizes with a probe or primer. The conditions under which hybridization occurs may vary with the size and sequence of the probe and the target sequence. By way of illustration only, a hybridization experiment can be performed by hybridization of a DNA probe (for example, a probe derived from the LTR of MLV labeled with a radioactive isotope) to a target nucleic acid electrophoresed in an agarose gel and transferred to a nitrocellulose membrane by Southern blotting (Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). Further discussion of hybridization conditions are given below in EXAMPLE 14.

In another embodiment, the target nucleic acid, upon hybridization to a therapeutically effective antisense oligonucleotide or oligonucleotide analog, results in the inhibition of expression of the target sequence. Either an antisense or a sense molecule can be used to target a portion of dsDNA, since both will interfere with the expression of that portion of the dsDNA. The antisense molecule can bind to the plus strand, and the sense molecule can bind to the minus strand. Thus, target nucleic acids can be ssDNA, dsDNA, and RNA.

Therapeutically Effective Amount: An amount sufficient to achieve a desired biological effect. In one embodiment, it is an amount effective to allow a functional level of expression of a nucleic acid, for example a gene, of interest. In particular examples, it is a concentration of retrovirus or retroviral vector with an altered host range effective to allow expression of the transgene, the expression of which is desired in a subject, sufficient to achieve a desired effect in the subject. For instance, it can be an amount necessary to improve signs and/or symptoms of a disease, for example by expression of one or more transgenes in the retroviral vector. Diseases include, but are not limited to, a neurological, immunological, cardiovascular, muscular, cell proliferative, or genetic disorder.

In another embodiment, it is an amount effective to inhibit expression of a nucleic acid, for example a gene of interest. In particular embodiments, it is a concentration of retrovirus or retroviral vector with an altered host range effective to allow expression of a therapeutically effective oligonucleotide, the expression of which is desired in a subject, sufficient to achieve a desired effect in the subject. For instance, it can be an amount necessary to improve signs and/or symptoms a disease, for example by expression of one or more therapeutically effective oligonucleotide in the retroviral vector. Complete inhibition is not necessary for therapeutic effectiveness. Therapeutically effective oligonucleotides are characterized by their ability to inhibit the expression of the gene of interest. Inhibition is any reduction in expression seen when compared to production in the absence of the oligonucleotide or oligonucleotide analog. Additionally, some oligonucleotides will be capable of inhibiting the expression of a gene of interest by at least 15%, 30%, 40%, 50%, 60%, or 70%, or more. Therapeutically effective oligonucleotides are additionally characterized by being sufficiently complementary to nucleic acid sequences encoding a gene of interest. As described herein, sufficiently complementary means that the therapeutically effective oligonucleotide can specifically disrupt the expression of a gene, and not significantly alter the expression of other genes.

An effective amount of retroviral vector having an altered host range may be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount of retroviral vector having an altered host range will be dependent on many factors, including, but not limited to: the retroviral vector having an altered host range administered; the subject being treated; the condition of the subject being treated; the severity and type of the condition being treated; the body weight or surface area of the subject to be treated; the age, weight, and sex of the subject to be treated; as well as the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a particular subject, and the manner of administration of the retroviral vector having an altered host range.

The general term "subject being treated" is understood to include all animals (e.g. humans, apes, dogs, cats, horses, and cows) that require expression of a transgene by a retroviral vector having an altered host range.

Therapeutically effective dose: A dose sufficient to allow functional expression of the transgene, resulting in a desired effect in a subject being treated, or which is capable of relieving signs or symptoms caused by the condition.

Therapeutic polypeptide: A polypeptide which can be used to alleviate or relieve a symptom of a disorder in a subject being treated. Specific, non-limiting examples of therapeutic polypeptides are cytokines or immunomodulators, hormones, neurotransmitters, or enzymes. In yet another embodiment, a therapeutic polypeptide is an immunogenic polypeptide.

Transduced and Transformed: A virus or vector transduces a cell when it transfers nucleic acid into the cell. A cell is "transformed" by a nucleic acid transduced into the cell when the DNA becomes stably replicated by the cell, either by incorporation of the nucleic acid into the cellular genome, or by episomal replication. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Transgene: An exogenous nucleic acid sequence, for example a gene sequence. In one embodiment the transgene encodes a marker protein which can be detected using methods known to one of skill in the art. Specific non-limiting examples of a maker protein include luciferase, GFP, and β-galactosidase. In another embodiment, the transgene encodes a therapeutic protein which can be used to alleviate or relieve a symptom of a disorder. Specific, non-limiting examples of therapeutic proteins include cytokines, immunomodulators, hormones, neurotransmitters, and enzymes. In another embodiment, the transgene encodes a therapeutically effective oligonucleotide, for example an antisense oligonucleotide, wherein expression of the oligonucleotide inhibits expression of a target nucleic acid sequence. In a further embodiment, the transgene encodes an antisense nucleic acid or a ribozyme.

The transgene can have the native regulatory sequences operably linked to the transgene (e.g. the wild-type promoter, found operably linked to the gene in a wild-type cell). Alternatively, a heterologous promoter can be operably linked to the transgene. In yet another embodiment, a viral LTR can be used to express the transgene.

Transgenic Cell: Transformed cells which contain foreign, non-native DNA.

U3: A non-coding region of a LTR about 200-1,200 nucleotides in length, located upstream of the transcription start site. Forms the 5' end of the provirus after reverse transcription and contains the promoter elements responsible for transcription of the provirus.

U5: A non-coding region of the LTR about 75-250 nucleotides in length. It is the first part of the genome to be reverse transcribed, forming the 3' end of the provirus genome.

Variant sequences: A variation of a nucleic acid sequence is a nucleic acid sequence having one or more nucleotide substitutions, one or more nucleotide deletions, and/or one or more nucleotide insertions, so long as the variant nucleic acid sequence substantially retains the activity of the original nucleic acid sequence, or has sufficient complementarity to a target sequence.

A variant nucleic acid sequence can also hybridize with the target DNA or RNA , under stringency conditions as described above. A variant nucleic acid sequence also exhibits sufficient

complementarity with the target DNA or RNA of the original oligonucleotide or analog as described above.

The present disclosure utilizes standard laboratory practices for the cloning, manipulation and sequencing of nucleic acids, purification and analysis of proteins and other molecular biological and biochemical techniques, unless otherwise stipulated. Such techniques are explained in detail in standard laboratory manuals such as Sambrook *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989), Coffin *et al.* (*Retroviruses*, Cold Spring Harbor Laboratory Press, 1997) and Ausubel *et al.*, *Current Protocols in Molecular Biology,* Greene Publishing Associates and Wiley-Intersciences (1987).

Identifying Host Range Control Elements (HRCEs)

A method is disclosed for identifying a HRCE in any retrovirus or retroviral vector. In one embodiment the retroviral vector is a simple retroviruses. In another example, the retroviral vector is a mammalian or avian retroviral vector, for example an ASLV or MLV ampho.

In one example, a heterogeneous population of nucleic acid sequences, such as a library, can be prepared (or purchased from a commercial source) from a retroviral vector of interest whose HRCE is yet unidentified. For example, a library can be prepared by isolating nucleic acid from a retroviral vector, and digesting the nucleic acid with one or more restriction enzymes, using methods known to one skilled in the art (Sambrook *et al.* In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). The heterogeneous population of nucleic acid sequences is inserted into a second retroviral vector whose HRCE is functionally deleted. This functional deletion can be achieved by directly inserting the heterogeneous population of nucleic acid sequences into the HRCE of the second retroviral vector, for example using standard cloning techniques. This results in the production of a chimeric retroviral vector (referred to as the chimeric vector), because it contains nucleic acid sequences from more than one retrovirus. The chimeric vector is tranfected into host cells of the second retrovirus, to determine if any heterogeneous nucleic acid sequence of the retrovirus whose HRCE is yet unidentified, can restore the functionally-deleted HRCE of the second retrovirus. The cells transfected are host cells of the second retrovirus. If any heterogeneous nucleic acid fragment of the retrovirus whose HRCE is yet unidentified restores the functionally deleted HRCE of the second retrovirus, the chimeric vector will be replication competent in the host cells, for example as determined by RT activity (see EXAMPLE 2) or any method that demonstrates the virus is replicating, for example monitoring the passages of a marker gene, such as an antibiotic resistance gene, or other cellular marker such as GFP or alkaline phosphatase.

To identify the sequence of the nucleic acid sequence present in the chimeric vector that restored the functionally-deleted HRCE of the second retrovirus, standard sequencing methods can be used, for example dideoxy sequencing, dye terminator sequencing, or direct sequencing using PCR (Sambrook *et al.* In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York,

1989). The insert is the newly identified HRCE of the retrovirus or retroviral vector whose HRCE was before unknown.

If no heterogeneous nucleic acid fragment of the retrovirus whose HRCE is yet unidentified can restore the functionally deleted HRCE of the second retrovirus, the chimeric vector will not be replication competent in the host cells. In this case, a new preparation of heterogeneous nucleic acid fragments can be prepared and tested (for example by using a library prepared with a different restriction enzyme).

In another example, a chimeric vector can be transfected into non-host cells to identify additional HRCEs in the second retrovirus. If the chimeric vector has a poor replication competency in non-host cells, but allows for the production of some virions, HRCEs of the second retrovirus can be identified by performing short and/or long-term adaptations of the chimeric vector by passaging the virions between host cells and non-host cells until a population of chimeric vector is obtained that is replication competent in the non-host cells. In one embodiment, a short-term adaptation is performed over a few passages, for example at least 10 passages. In another embodiment, a long-term adaptation is performed over several passages, for example at least one year or at least 50 passages. To identify the nucleic acid sequence(s) present in the chimeric retrovirus that effectuated the alteration in host range of the second retrovirus, the retrovirus obtained after adaptation can be sequenced using standard methods known to those skilled in the art as described above. By comparing adapted sequence to the wild-type (or starting) sequence of the second retrovirus, mutations are identified which function as HRCEs in the second retrovirus.

The host cells of the present disclosure include any cell from any organism in which a retrovirus is replication competent. Cells can be obtained directly from a sample from a subject, such as blood cell. Alternatively, cells are obtained from a commercial source, such as the American Type Culture Collection in Manassas, VA. In one example, a host cell is a neural cell, a vascular cell, a bone cell, a muscle cell, a tumor cell, a cancer cell, an immunological cell, an epidermal cell, a lung cell, a kidney cell, a cervical cell, a spleen cell, or a bone marrow cell.

Therefore, using the methods described herein, nucleic acid sequences suspected of being a HRCE can be tested, by determining if such sequences can rescue a retrovirus or retroviral vector having a functionally deleted HRCE.

Use of Newly-Identified HRCEs to Alter Host Range

A method is disclosed for using HRCEs to alter the host range of a retroviral vector. In one example, the HRCE is not an envelope sequence, or a portion thereof. In another example, the HRCE affects RNA structure, such as RNA primary, secondary, tertiary, or quarternary structure. In one specific example, the HRCE affects RNA secondary structure. One specific example is described below, wherein methods were used to generate and identify ASLV vectors having an expanded host range and MLV ampho vectors having a contracted host range. One skilled in the art will understand

that the examples provided herein can be used to manipulate the HRCE in any retrovirus of interest, for example any simple retrovirus.

For example, the host-range of any simple retrovirus, such as ASLV, MLV, SNV, MMTV or MPMV can be altered using the methods provided herein. Once the HRCE(s) of a retroviral vector of interest is identified, the host range can be controlled by functionally deleting the HRCE of the retroviral vector, for example using standard methods known to one skilled in the art (Sambrook *et al.* In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). In another embodiment, a functionally deleted HRCE can be replaced with another HRCE, for example an HRCE from another species of retroviral vector, to alter the host range of the retroviral vector of interest. The choice to generate a retroviral vector with an expanded or contracted host range will depend on the purpose for which the retrovirus is used. For example, to generate a mammalian retroviral vector for use in a mammalian cell it is of interest to have a retroviral vector with a contracted host range, such that the retroviral vector is no longer replication competent in a mammalian cell. In one example, the host range of a mammalian retroviral vector is contracted such that it is replication defective in a mammalian cell, such as any cell from a mammalian subject, such as a mouse, human, or rat. To develop an animal model for a human disease, the host range of the retroviral vector can be expanded. In one example, the host range of an avian retroviral vector is expanded such that it is replication competent in a mammalian cell, such as a human, mouse, rabbit, pig, or rat cell.

In yet another embodiment, as an alternative to deleting an entire HRCE sequence, a portion of the sequence can be deleted, as long as such a partial deletion destroys the function of the HRCE. In one embodiment, at least 50%, 60% or even 70% of an HRCE is deleted. In addition, the present method can be practiced by inactivating a HRCE using site-directed mutagenesis, for example using the techniques of linker-insertion, generation of nested sets of deletion mutants or cleavage of double-stranded closed circular DNA with pancreatic DNAse I. Non-site directed mutagenesis can be used to inactivate a HRCE, for example by using transposon mutagenesis with Tn5. This method is useful for detecting and functionally deleting HRCE that lack the general characteristics of the currently characterized HRCEs. In another embodiment, the present method can be practiced by leaving in an HRCE and ligating into that HRCE sequence another sequence, such as another HRCE sequence derived from another retroviral vector.

A method for generating a retroviral vector having an altered host range by functionally deleting a HRCE in a first retroviral vector, wherein the HRCE is not an envelope sequence or portion thereof is disclosed. In one example, the method further includes replacing the functionally-deleted HRCE with an HRCE from another, second, retroviral vector, thereby generating a chimeric retroviral vector. In yet another example, the host range of the second retroviral vector differs from the host range of the first retroviral vector.

The functionally-deleted HRCE can include a portion of a U3 region of an LTR. In one embodiment, the U3 region includes at least 15 or at least 96 nucleotides but no more than the entire

LTR length, such as 614 contiguous nucleotides of the MLV ampho LTR. In one example, the functionally-deleted HRCE includes at least 50 nucleotides having at least 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 1, 2, or 3. In another example, the functionally-deleted HRCE contains SEQ ID NO: 1, 2 or 3. Alternatively, the functionally-deleted HRCE contains at least 50 nucleotides, such as at least about 60, 70, 80, 90, 96, 100, 250, or 600 nucleotides, that hybridize with a complement of a SEQ ID NO:1, 2 or 3, wherein hybridization conditions include wash conditions of 0.1 X SSC, 0.5% SDS at 62°C.

In other examples, the HRCE of the second retroviral vector is a DR or portion thereof. For example, the HRCE of the second retroviral vector can have at least 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 1, 2 or 3. Alternatively, the HRCE of the second retroviral vector contains SEQ ID NO:1, 2, or 3. In yet another example, HRCE of the second retroviral vector has at least 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 11 or 13. Alternatively, the HRCE of the second retroviral vector contains SEQ ID NO:11 or 13. The maximum length of nucleic acid sequence that can be added to a retrovirus will depend on the virus, and can be determined using standard methods known to those skilled in the art. For example, the maximum nucleic acid sequence length that can be inserted into ASLV is about 2.5 kb, but is shorter for MLV.

Also disclosed is a method of selecting for a retroviral vector having an altered host range over a short or long-term adaptation period. In one example, a short-term adaptation period is about at least 10 passages, and a long-term adaptation period is about at least 50 passages of the retroviral vector. This method for generating a retroviral vector having an altered host range includes functionally deleting a HRCE in a first retroviral vector with a first host cell range, and the HRCE is not an envelope sequence or portion thereof. A nucleic acid sequence from a second retroviral vector with a second host cell range is incorporated into first retroviral vector generating a third retroviral vector, which is used to transfect a third host cell. Cell free supernatant containing retroviral particles from the transfected third host cell is collected and used to infect a fourth host cell. A cell-free supernatant containing retroviral particles is collected from the fourth host cell. In one example, this method is repeated to select a population of retroviral particles that is replication competent in the first host cell. In another example, the third and first host cell are from the same species and the fourth and second host cell are from the same species. In yet another example, the third and second host cell are from the same species and the fourth and first host cell are from the same species.

A method for generating an ASLV that is replication competent in mammalian cells is disclosed. The method includes functionally deleting a DR in ASLV, incorporating a nucleic acid sequence from a mammalian retroviral vector, such as MLV ampho, generating a chimeric retroviral vector, transfecting an avian cell, such as a chicken cell, with the chimeric retroviral vector and collecting a cell-free supernatant containing chimeric retroviral particles from the transfected avian cell. The cell-free supernatant containing the chimeric retroviral particles is then used to infect a mammalian cell, such as a human cell. A cell-free supernatant containing chimeric retroviral particles

from the mammalian cell is collected. This process can be repeated to select a chimeric retroviral vector that is replication competent in a mammalian cell.

Also encompassed by the disclosure is a retroviral vector, and a retroviral vector incorporated in a retroviral particle, having an altered host range obtained using the methods disclosed herein. In one example, the retroviral vector is no longer replication competent, it is replication defective, in at least one native cell. For example, a retroviral vector that is not replication competent in a mammalian cell.

Infection of Cells with Retroviral Vectors

A cell infected with a retroviral vector described above. In one example, the cell is a mammalian or avian cell, such as a human cell or chicken cell, such as a non-human mammalian cell, such as a mouse cell. In one embodiment, a non-human mammalian cell is infected with any retroviral vector disclosed herein and the retroviral vector is integrated into the genome of the non-human mammalian cell, using methods known to one skilled in the art (Sambrook *et al.* In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

A method is disclosed for transforming a cell *in vitro* or *in vivo* by contacting a cell with a retrovirus comprising a retroviral vector of the present disclosure including a transgene, where contact results in transducing the cell. A retroviral vector stably integrates into the cellular genome once it is introduced into cells. In one example, the retroviral vector is replication defective. In another example, the DNA of a retroviral vector is integrated into a chromosome of the cell. In another example, the retroviral vector contains a promoter operably linked to a transgene, such as a marker polypeptide or a therapeutic polypeptide. In yet another example, no other viral vector, such as a helper vector, is introduced into the cell. This expression can be used to alleviate the symptoms of or even treat a disease.

Therapeutic Uses of Retroviral Vectors with an Altered Host Range

The disclosed retroviral vectors can be used for introducing a nucleic acid sequence into a cell, using methods known to those skilled in the art (Sambrook *et al.* In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). The transfer of nucleic acids, such as genes, into cells provides a means to determine gene function. Alternatively, the transfer of nucleic acids can be used to treat diseases of a genetic basis. In addition, gene transfer provides the basis for high-level protein expression, used by molecular researchers to study protein function and to produce new protein drugs. The introduction of genes into animals can also produce useful animal models of human diseases.

In one example, mammalian retroviral vectors have a contracted host range, such that they are no longer replication competent in mammalian host cells, provide a safer vector for expression of a transgene in a mammalian cell, such as a mouse or human cell. Alternatively, retroviral vectors have an expanded host range, for example when the host-range of an avian retrovirus is expanded

such that it is replication competent in non-host mammalian cells, can be used to generate animal disease models.

A method for preventing, alleviating the symptoms of, or treating a disease in a subject is also disclosed. The method includes introducing into a subject's cell a therapeutically effective amount of a retroviral vector disclosed herein, wherein the vector contains a transgene, the cell is unable to produce viral particles, the introduction results in the stable genetic transduction of the cell and expression of the transgene, and the expression of the transgene results in alleviating a symptom of the disorder or preventing the disorder. Disorders include, but are not limited to neurological, immunological, cardiovascular, muscular, cell proliferative, or genetic disorders. The expression vector can be introduced into a subject's cells *ex vivo* and the cells reintroduced into the subject. Subjects of the present disclosure include mammals, such as humans and mice.

In another example, the method for treating a subject involves contacting a cell of the subject with a therapeutically effective amount of any retroviral vector disclosed herein, that is replication-defective and includes a transgene. Contact results in the retroviral vector integrating into a chromosome of the cell and expressing the transgene in the cell, wherein the cell is not contacted with any other virus, and the expression of the transgene treats the subject. In one embodiment the transgene is a therapeutic polypeptide or an antisense sequence.

Pharmaceutical compositions containing a retroviral vector with an altered host range, wherein the vector contains a transgene, and a pharmaceutically acceptable carrier are disclosed.

The retroviral vectors disclosed herein can be used for short-term (for example for immunization) and long-term (for example for gene replacement therapy for missing or defective genes) expression of a transgene. The vectors can be used to deliver an immunogen to achieve an improved CTL response.

Testing Retroviral Vectors with an Altered Host Range in Disease Models

The retroviral vectors having an altered host range described herein can be tested for their ability to express a transgene *in vivo* using mouse models which have been generated for various diseases. Mice which are functionally deleted for a gene are infected with a retroviral vector having an altered host range containing the missing gene. Mice are then screened for their ability to express the missing gene as a transgene, and for the ability of the transgene to correct the phenotypic affect of the transgene deletion.

Use of Retroviral Vectors with an Altered Host Range to Disrupt Gene Expression

A retroviral vector having an altered host range which includes an antisense molecule as the transgene is disclosed. In general, the antisense molecule binds complementarily to the target nucleic acid. Complementary binding occurs when the base of one molecule forms a hydrogen bond with another molecule. Normally the base adenine (A) is complementary to thymidine (T) and uracil (U), while cytosine (C) is complementary to guanine (G). Therefore, the sequence 5'-TCGT-3' of the

antisense molecule will bind to ACUC of the target RNA, or 5'-ACTC-3' of the target DNA. Additionally, in order to be effective, the antisense and sense molecules do not have to be 100% complementary to the target RNA or DNA.

To design antisense oligonucleotides, the mRNA sequence from a desired gene is examined. Regions of the sequence containing multiple repeats, such as TTTTTTTT, are not as desirable because they will lack specificity. Several different regions can be chosen. Of those, oligonucleotides are selected by the following characteristics: those having the best conformation in solution; those optimized for hybridization characteristics; and those having less potential to form secondary structures. Antisense molecules having a propensity to generate secondary structures are less desirable.

Antisense nucleic acids are polynucleotides, and can be oligonucleotides (ranging from 6 to about 100 oligonucleotides). In specific aspects, the oligonucleotide is at least about 10, 15, or 100 nucleotides, or a polynucleotide of at least 200 nucleotides. An antisense nucleic acid can be much longer. Generally, a longer complementary region will give rise to a molecule with higher specificity. When retroviral vector having an altered host range is introduced into a cell, the cell supplies the necessary components for transcription of the therapeutic antisense molecule.

The nucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, and can include other appending groups such as peptides, or agents facilitating transport across the cell membrane (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* 1989, 86:6553-6; Lemaitre *et al.*, *Proc. Natl. Acad. Sci. USA* 1987, 84:648-52; PCT Publication No. WO 88/09810) or blood-brain barrier (PCT Publication No. WO 89/10134), hybridization triggered cleavage agents (Krol *et al.*, *BioTechniques* 1988, 6:958-76) or intercalating agents (Zon, *Pharm. Res.* 1988, 5:539-49).

The antisense polynucleotide can be modified at any position on its structure with substituents generally known in the art. For example, a modified base moiety can be 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N~6-sopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, methoxyarninomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-S-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine.

In another example, the polynucleotide includes at least one modified sugar moiety such as arabinose, 2-fluoroarabinose, xylose, and hexose, or a modified component of the phosphate backbone, such as phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a

phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, or a formacetal or analog thereof.

Catalytic nucleic acid and other oligomeric molecules can be designed which degrade target sequences and included in a retroviral vector having an altered host range of the disclosure. Such catalytic antisense molecules can contain complementary regions that specifically hybridize to the target sequence, and non-complementary regions which typically contain a sequence that gives the molecule its catalytic activity. Conjugates of antisense with a metal complex, e.g. terpyridylCu (II), capable of mediating mRNA hydrolysis, are described in Bashkin *et al.*, *Appl. Biochem Biotechnol.* 1995, 54:43-56.

A particular type of catalytic nucleic acid antisense molecule is a ribozyme or anti-sense conjugates, which may be used to inhibit gene expression (PCT publication WO 9523225, and Beigelman *et al. Nucl. Acids Res.* 23:4434-42, 1995). Examples of oligonucleotides with catalytic activity are described in WO 9506764, WO 9011364, and Sarver *et al.*, Science 247:1222-5, 1990.

The relative ability of an oligomer such as a polynucleotide to bind to a complementary strand is compared by determining the T_m of a hybridization complex of a polypeptide and its complementary strand. The T_m, a characteristic physical property of double helices, denotes the temperature in degrees Centigrade at which 50% helical versus coiled (unhybridized) forms are present. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). A reduction in UV absorption indicates a higher T_m. The higher the T_m the greater the strength of the binding of the hybridized strands. As close to optimal fidelity of base pairing as possible achieves optimal hybridization of a polynucleotide to its target RNA.

Methods for *in vivo* Transgene Expression using Retroviral Vectors with an Altered Host Range

A method is disclosed for expressing a transgene *in vivo* using a retroviral vector having an altered host range. Retroviruses can be used for *in vivo* gene expression because they have a high efficiency of infection and stable integration and expression (Orkin *et al.*, 1988, *Prog. Med. Genet.*7:130-42). In one example, the method is a method for combating chronic infectious diseases, such as HIV, as well as non-infectious diseases such as cancer and birth defects such as enzyme deficiencies in a subject.

In one example, retroviral vectors having an altered host range are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. In a particular example, the structural genes (i.e., *gag*, *pol*, and *env*), are removed from the retroviral backbone using known genetic engineering techniques. Examples include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal. The transgene(s) of interest can be incorporated into the proviral backbone in several ways. In the most straightforward constructions, the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the LTR.

Retroviral vectors have also been constructed which can introduce more than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter. Alternatively, two genes may be expressed from a single promoter by the use of an Internal Ribosome Entry Site (IRES).

Cells can be removed from a subject having deletions or mutations of a gene, and then a retroviral vector having an altered host range (containing the therapeutic transgene) is introduced into the cell. These transfected cells will thereby produce functional transgene protein and can be reintroduced into the patient. Methods described in U.S. Patent No. 5,162,215 (Bosselman *et al.*) teach how to detect the presence and expression of a gene of interest in target cells. Methods described in U.S. Patent No. 5,741,486 (Pathak *et al.*) teach the use of viral vectors in gene therapy. Such methods can be applied to the retroviral vectors having an altered host range of the present disclosure, for example in *in vivo* expression of a transgene.

In addition, the retroviral vectors having an altered host range can be introduced into a subject *in vivo*. The scientific and medical procedures required for mammalian cell transfection are now routine. For example, immunotherapy of melanoma patients using genetically engineered tumor-infiltrating lymphocytes (TILs) has been reported by Rosenberg *et al.* (*N. Engl. J. Med.* 323:570-8, 1990). There, a retroviral vector was used to introduce a gene for neomycin resistance into TILs. A similar approach can be used to introduce a transgene into a subject using the retroviral vectors disclosed herein.

In some embodiments, the present disclosure relates to a method of treating subjects which underexpress a gene, or in which greater expression of a gene is desired. These methods can be accomplished by introducing a transgene coding for the underexpressed gene into retroviral vectors having an altered host range, which is subsequently introduced into the subject.

In some of the foregoing examples, it may only be necessary to introduce the genetic or protein elements into only certain cells or tissues. However, in some instances (i.e. tumors), it is more therapeutically effective and simple to treat all of the subject's cells, or more broadly disseminate the vector, for example by intravascular administration.

The retroviral vectors having an altered host range can be administered to a subject by any method which allows the vectors to reach the appropriate cells. These methods include injection, infusion, deposition, implantation, or topical administration. Injections can be intradermal or subcutaneous.

In addition, retroviral vectors having an altered host range can be designed to use different promoters to express the transgene. In one embodiment, a retroviral LTR sequence can serve as a promoter for expression of the transgene. Thus, in one example, a therapeutic nucleic acid is placed under the control of a retroviral LTR promoter. In another embodiment, the transgene is operatively linked to a heterologous promoter (e.g. the CMV promoter). In yet another example, the transgene is operatively linked to a tissue specific promoter (e.g. the immunoglobulin promoter), such that the

expression of the transgene occurs only in a tissue of interest. Other suitable promoters which may be employed include, but are not limited to, the gene's native promoter, any retroviral LTR promoter such as the RSV promoter; inducible promoters, such as the MMTV promoter; the metallothionein promoter; heat shock promoters; the albumin promoter; the histone promoter; the α-actin promoter; TK promoters; B19 parvovirus promoters; and the ApoAI promoter. However the scope of the disclosure is not limited to specific transgenes or promoters

Ex Vivo Transfection of Cells

Ex vivo methods for introducing a retroviral vector having an altered host range involve removing a cell or tissue (such as a graft) from a subject and subsequently transducing the cell *ex vivo*, and then introducing the cell into the subject. For example, retroviral vectors having an altered host range can be used to treat autologous cells isolated from a subject. In one example, the cells are obtained or cultured from a subject such as lymphocytes, macrophages or stem cells. Alternatively, the cells can be heterologous cells such as those stored in a cell bank (e.g., a blood bank).

In one embodiment, the cells are T cells. Several techniques are known for isolating T cells. In one method, Ficoll-Hypaque density gradient centrifugation is used to separate PBMC from red blood cells and neutrophils according to established procedures. Cells are washed with modified AIM-V (which consists of AIM-V (GIBCO) with 2 mM glutamine, 10 μg/ml gentamicin sulfate, 50 μg/ml streptomycin supplemented with 1% FBS). Enrichment for T cells is performed by negative or positive selection with appropriate monoclonal antibodies coupled to columns or magnetic beads according to standard techniques. An aliquot of cells is analyzed for desired cell surface phenotype (e.g., CD4, CD8, CD3, CD14, etc.). Transduced cells are prepared for reinfusion according to established methods. See, Abrahamsen *et al., J. Clin. Apheresis* 6:48-53, 1991; Carter *et al., J. Clin. Arpheresis* 4:113-7, 1988; Aebersold *et al., J. Immunol. Methods* 112:1-7, 1988; Muul *et al., J. Immunol. Methods* 101:171-81, 1987; and Carter *et al., Transfusion* 27:362-5, 1987).

In another example, retroviral vectors having an altered host range can be used to treat a heterologous graft which is then transplanted into a subject. For example, retroviral vectors having an altered host range can be used to infect a liver, which is subsequently transplanted into a subject requiring a liver transplant. Alternatively, the graft can be a bone marrow graft, lung graft, heart graft, kidney graft, bone graft, or vascular graft.

In Vivo Transfection of Cells

Retroviral particles containing a retroviral vector having an altered host range including a transgene encoding a therapeutic protein can be administered directly to a subject for transduction of cells *in vivo*. Administration is by any of the routes normally used for introducing a molecule into cells. The packaged nucleic acids are administered in any suitable manner, such as with pharmaceutically acceptable carriers. Suitable methods of administering such packaged nucleic acids in the context of the present disclosure to a subject are available, and although more than one route

can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

In determining the effective amount of a retroviral vector having an altered host range to be administered in the treatment of a disease, the physician or other clinician evaluates symptom or clinical parameters, including the progression of the disease (and other factors listed above). In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 μg to 100 μg for a typical 70 kilogram subject.

EXAMPLE 1

Cell Culture and Transfection

CEF cells are non-transformed chicken fibroblast cells that can be used as host cells to grow avian-derived retroviruses, and can be passaged up to about 30 times. CEF cells were cultured from 11-day embryos of "line 0" chickens (Whitcomb *et al., J. Virol.* 69:6228-38, 1995) and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, BRL) supplemented with 5% fetal bovine serum (FBS), 5% newborn calf serum (NBS), 3% tryptose phosphate broth, 100 U/ml penicillin and 100 μg/ml of streptomycin. DF-1 cells, a continuous non-transformed cell line derived from EV-0 chicken embryo fibroblasts which are permissive for RCASBP viruses (Himley *et al., Virology* 248:295-304, 1998; Schaefner-Klein *et al., Virology* 248:305-11, 1998), were cultured identically to CEF cells.

Other suitable host cells for avian-derived retroviruses include any avian derived cells, including, but not limited to: QT6, a chemically transformed quail fibroblast cell line (American Type Culture Collection (ATCC), Manassas, VA #CRL-1708) chicken bursa lymphoblast cells (ATCC #CRL-2112 and 2111), turkey cells (ATCC #CRL-1835), duck cells (ATCC #CCL-141) as well as any cultured, transformed or non-transformed avian cell, such as chicken fibroblast cells, chicken liver cells or chicken epidermal cells.

The human embryonic kidney cell line 293 can be used as host cells to grow mammalian-derived retroviruses, such as MLV. The promoter in the ALV LTR is efficiently transcribed in 293 cells. The 293 cells were grown in D-MEM supplemented with 5% calf serum, 5% fetal calf serum (FCS) and Pen-Strep. The 293R(A) and 293R(B) cells are puromycin-resistant 293 cells stably transfected with the gene for either the ALV subgroup A (tva) or B (tvb) receptors. The 293R cells were generated as follows: 293 cells (ATCC, #CRL-1573) were transfected using $CaPO_4$ with 2 μg of the plasmid pPur encoding puromycin-N-acetyl-transferase (Clonetech) and 20 μg of plasmid pKZ261 (a pCB6 expression plasmid encoding a synthetic Tva gene, Belanger *et al., J. Virol.* 69:1019-24, 1995) or 20 μg of plasmid pBK7.62 (an expression plasmid encoding a synthetic Tvb gene, Brojatsch *et al., Cell*, 87:845-55, 1996). Transfected cells were selected after 24 hours with medium containing 0.5 μg/ml puromycin. 293R(A) and (B) cells express the avian receptors at high levels and can be infected with subgroup (A) and (B) ASLVs, respectively. The growth medium for

these cells contains 1 μg/ml puromycin which helps maintain the receptor genes. Cells were passaged using Trypsin-DeLarco medium (Quality Biological, Inc., MD).

The 3T3 cell line is a continuous cell line established from disaggregated Swiss mouse embryo cultures. The 3T3 cells were grown in DMEM supplemented with 5% calf serum, 5% FCS and Pen-Strep.

Other mammalian cells which can be used the practice the disclosed method include, but are not limited to HeLa cells, SW-527 human cells (ATCC #7940), WISH cells (ATCC #CCL-25), Daudi calls (ATCC #CCL-213), Mandin-Darby bovine kidney cells (ATCC #CCL-22) and Chinese Hamster ovary cells (ATCC #CRL-2092).

Yeast cells that can be used include *Pichia pastoris* (ATCC #201178) and *S. cerevisiae* (ATCC #46024). Insect cells include cells from *D. melanogaster* (ATCC #CRL-10191), the cotton bollworm (ATCC #CRL-9281) and from *Trichoplusia ni* egg cell homoflagelates. Fish cells that can be used include those from rainbow trout (ATCC #CLL-55), salmon (ATCC #CRL-1681) and Zebrafish (ATCC #CRL-2147). Amphibian cells that can be used include those of the Bullfrog, *Rana catesbelana* (ATCC #CLL-41). Reptile cells that can be used include those from Russell's Viper (ATCC #CCL-140). Plant cells that can be used include *Chlamydomonas* (ATCC #30485), *Arabidopsis* (ATCC #54069) and tomato (ATCC #54003).

Transfections were preformed using the calcium phosphate method of Wigler *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 76:1373-6, 1979) using 10 to 20 μg of Qiagen-purified DNA per 100 mm dish and using a glycerol shock four hours after the precipitate was added. The transfected cells were passaged to confluence. Prior to passage, the culture supernatant was collected, cleared by centrifugation at 1500 xg for 10 minutes and stored at -70°C for later analysis.

EXAMPLE 2

Generation of a Mini-Library of DR-deleted ASLV containing MLV ampho inserts

The RCAS vectors (replication competent ALV with a splice acceptor) are replication competent avian retroviral vectors based on RSV. RCAS was constructed by removing the *src* gene and the upstream direct repeat (DR) that lies between *env* and *src* from a molecularly cloned SR-A strain of RSV (DeLorbe *et al.*, *J. Virol.* 36:50-51, 1980; Hughes and Kosik, *Virology* 136:89-99, 1984). RCAS retains the *src* splice acceptor and the downstream DR. A unique ClaI site was inserted at the site of the *src* deletion to facilitate the cloning and expression of genes of interest. The RCASBP vectors contain the *pol* gene from the Bryan high titer strain of RSV (Sudol *et al.*, *Nucleic Acids. Res.* 14:2391-405, 1986; Petropoulos and Hughes, *J. Virol.* 65:3728-37, 1991).

A schematic diagram of the generation of a DR-deleted ASLV containing MLV inserts is shown in FIG. 1. A DR-deleted version of RCASBP(A) (A refers to the class of receptors used by the virus) was created by removing a 72-bp sequence (containing the conserved region of the DR) located between the unique MluI and Bsu36I sites from the 3′ UTR. This sequence is located between the *env* gene and the polypurine tract (ppt) and is required for normal viral growth. The resulting vector,

RCASBP(A)ΔDR, is replication defective in both avian and mammalian cells. To facilitate the cloning of blunt ended restriction fragments, RCASBPΔDR was linearized with ClaI and the ends of the DNA made blunt with T4 DNA polymerase and dephosphorylated with shrimp alkaline phosphatase (AP) (Boehringer Mannheim) to inhibit re-ligation of the vector.

An amphotropic murine leukemia virus (MLV ampho) genomic clone (pRR145: Ferris, Master's Thesis from Hood College entitled: Analysis of the avian sarcoma-leukosis virus DR, an RNA element essential for viral replication: Complementation and spontaneous mutations restore replication in a viral vector lacking the DR, 2000; Shinnick *et al.*, *Nature* 239:543-8, 1981; Ott *et al.*, *J. Virol.* 66: 6107-16, 1992), which grows efficiently in murine, avian, and human cells, was digested into small, blunt ended fragments using AluI, BstUI, and DpnI in three separate reactions. The resulting DNA fragments were ligated into the blunt ends of the ClaI cut, T4 DNA polymerase treated RCASBP(A)ΔDR. This library of chimeric vectors contains fragments of pRR145 in the 3' UTR of RCASBP(A)ΔDR at the site normally occupied by the DR. The ligation mixtures were used to transform *E. coli* DH5α competent cells (Life Technologies, Gaithersburg, MD). Individual bacterial colonies were recovered from plates, pooled, and grown together in liquid culture. Plasmid DNA was extracted and purified using the Qiagen maxi plasmid purification procedure (Qiagen, Inc., Valencia, CA). This DNA constituted a "mini-library" of DR-deleted ASLV-containing MLV inserts (referred to as RCASBP(A)ΔDRMLV).

EXAMPLE 3

Selection of Replication Competent Clones in Avian Cells

Removal of the conserved region of DR RCASBP results in a virus that can no longer replicate in chicken cells. To determine whether sequences from an amphotropic MLV genomic clone can functionally replace the DR sequence removed from the replication defective vector, RCASBP(A)ΔDR (FIG. 1), a selection method was developed.

The plasmid "mini library" of RCASBP(A)ΔDR containing MLV ampho inserts obtained in EXAMPLE 2 (RCASBP(A)ΔDRMLV) was transfected into avian DF-1 cells using $CaPO_4$ as described above in EXAMPLE 1. Transfected cells were passaged and viral growth monitored by assaying for reverse transcriptase (RT) activity in cell-free supernatants. The presence of RT activity demonstrates that the cells are transformed and the retroviral vector is replicating within the transformed cells. Culture supernatants were collected and centrifuged at low speed (3000 rpm) to remove cells and debris. Culture supernatant (1 ml) was centrifuged at 4°C for 30 minutes at the maximum speed in an EPPENDORF™ tabletop centrifuge. The supernatant was completely aspirated and 100 µl of reaction buffer (50 mM Tris, pH 8.0, 20 mM DTT, 12 mM $MgCl_2$, 60 mM NaCl, 0.1% NP-40, 0.25 U/ml rCdG template-primer, 0.1 mM dGTP, and 10 µCi ^{32}P-labeled dGTP/ml) added to the tube. Reactions were incubated for one hour at 37°C, combined with 200 µl of 0.2 M sodium pyrophosphate buffer containing salmon sperm DNA carrier, and TCA precipitated onto glass fiber filters. The reaction was assayed for RT activity by measuring the amount of ^{32}P-

labled dGTP incorporated onto acid-precipitable material, using an oligo(dG) primer and a poly(rC) template, using a Packard TriCarb scintillation acid-precipitable material.

At passage four, detectable levels of RT activity were observed. Infected cultures derived from the original transfections were continuously passaged. In addition, cell-free culture supernatants containing virus were used to infect fresh DF-1 cells. The chimeric virus, RCASBP(A)ΔDR containing MLV ampho inserts, was replication competent in DF-1 cells and transfer of viral supernatants to previously uninfected cells allowed for the selection of the most infectious (best-adapted) virus.

EXAMPLE 4

Identification of an MLV ampho Sequence that Promotes Viral Replication

To identify the MLV ampho sequence(s) that conferred to RCASBP(A)ΔDR the ability to be replication competent in avian cells, copies of the viral genome were obtained from infected cells. Unintegrated linear and circular DNA copies of the viral genome were present in the cytoplasm 36-48 hours after retroviral infection. These unintegrated viral DNAs were produced by infecting fresh DF-1 cells with the cell-free viral supernatant from a culture of DF-1 cells that had been transfected with RCASBP(A)ΔDRMLV (EXAMPLE 2) and was producing virus. Supernatants containing infectious virus were used to make Hirt DNA (Hirt, *J. Mol. Biol.* 26:365, 1967).

Following infection (36-48 hours), copies of the viral genome and other small DNAs were isolated, by lysing the cells in pH 7.5 buffer containing 0.6 % SDS, 10 mM EDTA and 50mM Tris. Following an overnight incubation at 4°C in the presence of 1 M NaCl, genomic DNA and SDS were removed by centrifugation. Small DNAs, including small circular viral genomes, are retained in the supernatant. The supernatant was extracted twice with phenol-chloroform, once with chloroform, and ethanol precipitated.

The resulting DNA was used as a substrate for specific amplification of viral sequences. To amplify the DNA sequences cloned into the *Cla* I site of the replicating virus, the following primers were used: 5'-GAGCTGACTCTGCTGGTGCC-3' (SEQ ID NO: 6) and 5'-CCCCCTCCCTATGCAAAAGCG-5' (SEQ ID NO: 7). These primers anneal to the RSV sequence on either side of the *Cla* I site. PCR conditions were 30 cycles of: 40 seconds at 90°C, 1 minute 20 seconds at 59°C, and 30 seconds to 1 minute at 72°C. The PCR product was gel purified and the region of the PCR fragment containing the MLV insert sequenced in both directions. Sequencing was performed using an automatic cycle sequencing machine and a PRISM™ READY REACTION™ dideoxy cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed with an automated 373A DNA sequencer (Applied Biosystems).

Alternately, PCR can be performed using crude cell lysates instead of preparing Hirt DNA. For example, two µl of the loose cell pellet is added to eight µl of a lysis buffer (1X PCR buffer with 0.1% Triton X-100). To disrupt the cells, the suspension is overlaid with 200 µl of paraffin oil and sonicated with a VIBRACELL ™ sonicator (Sonics and Materials, Inc., Danbury, Conn.) equipped

with a microprobe at level 3 for 15 seconds. The emulsion is centrifuged at the maximum speed in a refrigerated EPPENDORF™ tabletop centrifuge for 10 minutes to separate the phases, and 1.5 µl of the aqueous phase was used in the PCR reaction.

The MLV ampho insert that conferred to RCASBP(A)ΔDR the ability to be replication competent in avian cells, was about 200 nucleotides in length (the 196 bp sequence shown in SEQ ID NO: 2), and is referred to herein as the 200-bp insert. The sequence derived from a portion of the U3 region of the MLV LTR and is bordered by AluI recognition sites in the MLV genome but does not include the AluI sites (FIG. 2). The short sequences between the 200-bp insert and the AluI sites were apparently lost during cloning and selection.

EXAMPLE 5

RCASBP(A)ΔDRU3 is Replication Competent in Avian Cells

To prepare a molecularly cloned version of the chimeric virus, the 200-bp insert (SEQ ID NO: 2) identified in the replicating chimeric virus in EXAMPLE 4 was PCR amplified using pRR145 MLV as a template and the primers and PCR conditions described in EXAMPLE 4. The resulting product was ligated into RCASBPΔDR that had been cleaved with ClaI and treated with shrimp AP. The resulting vector, RCASBP(A)ΔDRU3, was transfected into DF-1 cells as described above.

RCASBP(A)ΔDRU3 required two viral passages in DF-1 cells before RT activity approached that of the wild-type RCASBP. Hirt DNA was prepared from cells infected with the passaged virus and the region containing the U3 insert PCR amplified and sequenced as described in EXAMPLE 4. Sequence results showed that the upstream half of the MLV U3 insert was lost during passage. As shown in FIG. 3, the insert sequence was reduced to 96-bp (SEQ ID NO: 1) and the 38-bp downstream flanking sequence (SEQ ID NO: 10) present in the original isolate was retained.

The experiment was repeated with constructs that included the flanking sequences at each end of the U3 fragment. To generate RCASBPΔ(A)DRU3F, the MLV U3 fragment and flanking sequences were PCR amplified from Hirt DNA isolated from DF-1 cells infected with RCASBP(A)ΔDRU3 using the methods described in EXAMPLE 4. PCR primers were used that anneal to the flanking sequences and encode ClaI sites at each end of the fragment (5' GGCATCGATCCTGAATGTGGGCCGGGC 3' (SEQ ID NO: 14) and 5' CGCATCGATCTGAATATGGGCCAAACAGG 3' (SEQ ID NO: 15)). The resulting fragment was digested with ClaI and ligated into RCASBP(A)ΔDR digested with ClaI and treated with shrimp AP.

RCASBPΔDRU3F also required two passages before wild-type levels of RT activity were observed. The passaged virus contained a deletion in the upstream portion of the U3 insert similar to the deletion in passaged RCASBPΔDRU3. RCASBPΔDR vectors containing the U3 fragment in the antisense orientation failed to replicate.

EXAMPLE 6

RCASBPΔDRMLV has an Altered Host Range

Wild-type avian retroviruses such as ASLV do not replicate in mammalian non-host cells. The viral envelope-cellular receptor interaction imposes the initial constraint on viral entry. For ASLVs with subgroup (A) or (B) envelopes, this block can be overcome by infecting mammalian cells that express the cloned avian subgroup A or B cellular receptors. Viral DNA can also be transfected directly into mammalian cells.

To determine whether substitution of MLV ampho sequences into RCASBP(A)ΔDR would enhance the ability of the virus to be replicate competent in mammalian cells, cell free supernatants from DF-1 cells transfected with RCASBP(A)ΔDRMLV (EXAMPLE 2) were used to infect mammalian 293 cells that express the avian subgroup A receptor, 293R(A) cells.

RCASBP(A)ΔDRMLV replicated, albeit poorly, in mammalian 293R(A) cells. In contrast to the original RCASBP(A) avian virus, transfection of the chimeric virus RCASBP(A)ΔDRMLV into mammalian cells resulted in the production of viral particles. Therefore, MLV ampho insert altered the host range of RCASBP by expanding the host range of RCASBP. Although 293R(A) cells produced only low amounts of infectious virus, the number of infectious virions was amplified by growing the virus in DF-1 cells. After one passage of the viral stock on 293R(A) cells, culture supernatants from infected 293R(A) cells were transferred back to DF-1 cells. Culture supernatants from these infected DF-1 cells were used to infect fresh DF-1 cells for the production of Hirt DNA.

The region containing the insert was amplified and sequenced as described in EXAMPLE 4. Half of the original U3 insert and the downstream flanking sequence were retained by the virus (SEQ ID NO: 1). Viral passage of cell-free supernatants between 293R(A) and DF-1 cells was used to select for an adapted virus that was replication competent in both cell types. After four viral passages between DF-1 and 293R(A) cells, Hirt DNA was prepared and the insert sequenced (see EXAMPLE 4). The portion of the RCASBP vector between *env* and the ClaI site and the first 100 bases of the original U3 fragment were deleted, reducing the insert to 96 bp (FIG. 3 and SEQ ID NO: 1). The 96 bp fragment corresponds to a part of the U3 region of the MLV ampho LTR, upstream of the "TATA" box.

Passage of the RCASBP(A)ΔDRU3F viral supernatants (see EXAMPLE 5) between DF-1 and 293R(A) cells selected the same deletion of the upstream half of the U3 insert. The downstream 96-bp of the U3 insert and about 38-bp downstream flanking sequence were retained (FIG. 3).

EXAMPLE 7

Generation and Expression of RCASBP(A)ΔDRTrU3F in Avian and Non-Avian Cells

The sequences retained in the U3 insert of the passaged virus were used to create a vector containing the 96-bp MLV U3 fragment (SEQ ID NO: 1) and the downstream flanking sequence (SEQ ID NO: 10).

A segment containing the 96-bp insert was amplified by PCR from Hirt DNA (see EXAMPLE 4) containing adapted RCASBPΔDRU3F, using primers that flank the unique upstream SalI and ClaI site (5'-ATGTTTCCAGGGTGCCCCAA-3' (SEQ ID NO: 4) and 5'-AGCAGAAGCGCGCGAACAGAA-3' (SEQ ID NO: 5)). Other primers can be used. Appropriate flanking primers are chosen using the sequence information in SEQ ID NO:1 and SEQ ID NO:3. Such primers flank the sequence to be synthesized, one primer complementary to the 3' - 5' strand and the other primer complementary to the 5' - 3' strand, and should be about 12 to 30 nucleotides long.

The amplified 96-bp fragment was ligated into the *Cla*I site of RCASBPΔDR. The resulting construct, RCASBP(A)ΔDRTrU3F, was transfected into DF-1 cells and produced levels of RT activity equivalent to RCASBP(A).

Cell free viral supernatants from DF-1 cells producing RCASBPΔDRTrU3F virus were used to infect 293R(A) cells. Low to no levels of RT activity were detected in 293R(A) culture supernatants and the infection did not spread in 293R(A) cell cultures, indicating that RCASBPΔDRTrU3F did not replicate in mammalian cells unlike adapted RCASBPΔDRU3F or adapted RCASBPΔDRMLV which were replication competent through at least one generation.

EXAMPLE 8

Mutations in *gag* Alter the Host Range

The inability of RCASBPΔDRTrU3F to replicate in 293R(A) cells indicated that during selection, changes occurred in the viral genome in areas other than the 3' UTR. Modifications in the *gag* gene were investigated. Gag proteins are involved in the specific packaging of viral RNA. Mutations that alter *gag* may affect the recognition or packaging of viral RNA by changing RNA structure or the amino acid sequence of gag.

Hirt DNA prepared from cells infected with virus from the uncloned RCASBPΔDRMLV stock that had been adapted and amplified on DF-1 and 293R(A) cells was used as the template for amplification of *gag*. Segments spanning SacI to SacII (using primers 5' CCGTCGGAGGGAGCTCCAGG 3' (SEQ ID NO: 16) and 5' GGGCTGGATAGCAGACGACATGG 3' (SEQ ID NO: 17)) and SacII to HpaI (using primers 5' CGGATCAAGGCATAGCCGCGGCC 3' (SEQ ID NO: 18) 5' GGCGCCCCCTGTTGGACGGCCCC 3' (SEQ ID NO: 19)) were amplified from the *gag* gene using the methods described in EXAMPLE 4. The wild-type *gag* gene was deleted from RCASBP(A)ΔDRTrU3F (EXAMPLE 7) digested with SacI and HpaI and replaced with the *gag* gene obtained from the adapted virus in a three piece ligation.

The resulting viral vector, RCASBP(A)ΔDRTrU3FNg, was transfected into DF-1 cells and wild type levels of RT activity were detected at an early cell passage. Cell-free supernatants from DF-1 cells producing RCASBP(A)ΔDRTrU3FNg were used to infect 293R(A) cells. In contrast to the results obtained with the RCASBP(A)ΔDRTrU3F virus with an unmodified *gag* gene, high levels

of RT activity were detected. Therefore, the host range of RCASBP(A) was altered by mutations in *gag* allowing the virus to be replication competent in mammalian cells.

Although RCASBP(A)ΔDRTrU3F and similar vectors containing wild-type *gag* sequences grew well on DF-1 cells, 293R(A) cells infected with these viruses did not produce detectable levels of RT activity (thus the virus was not replication competent in these cells). The sequence of *gag* derived from the adapted virus was compared to that in RCASBP(A)ΔDRTrU3F. Six point mutations were observed in *gag* derived from the short-term adapted virus (FIG. 4, SEQ ID NO: 11). As shown in Table 1 (mutations 1-6), four mutations resulted in silent changes (no change in the amino acid sequence), and two replaced Ala residues with a Val and Thr. Therefore, the changes in *gag* are likely to affect RNA structure and have less influence on the structure and function of gag proteins. Five mutations were located in the MA coding region and one was downstream from the end of MA (FIG. 4). No mutations were observed in other parts of *gag*.

Table 1: Summary of the point mutations in *gag*

Mutation Number	Nucleic Acid Mutation	Resulting Amino Acid Change
1	GCG to GTG	Ala to Val
2	GAT to GAC	Asp (no change)
3	GCA to ACA	Ala to Thr
4	GTT to GTC	Val (no change)
5	GAG to GAA	Glu (no change)
6	CAC to CAT	His (no change)
7	GGG to GAG	Gly to Glu acid
8	GTG to GTA	Val (no change)

Eight point mutations were acquired in *gag* of RCASBPΔDRMLVU3 during adaptations on DF-1 and 293R(A) cells. All nucleotide substitutions were transitions.

The vector RCASBP(A)ΔDRNg was constructed as a cloning intermediate. This construct includes the *gag* sequence derived from the short-term adapted virus (SEQ ID NO: 11) but contains no DR element. Transfection of RCASBP(A)ΔDRNg into DF-1 cells resulted in the production of a virus that replicated to about 20% of wild-type levels as measured by RT activity. The virus did not replicate in 293R(A) cells. Therefore, mutations in *gag* (SEQ ID NO: 11) cause structural changes in viral RNA which partially substitute for the DR, and influence viral replication.

RCASBP(A)ΔDRTrU3FNg was modified to produce RCASBP(A)ΔDRNg1-9. In the process of adaptation, the viral sequence between *env* and ClaI (which includes a splice acceptor) and the *env* sequences coding for the last three amino acids of the envelope transmembrane protein were lost. The virus included the entire *env*, the region containing the splice acceptor, and a unique Cla I site. The vector also contained a modified *gag* (SEQ ID NO: 11), as well as the 96-bp fragment from MLV (SEQ ID NO: 1) and 38-bp of downstream flanking sequence (SEQ ID NO: 10) in place of the deleted DR (FIG. 5A).

RCASBP(A)ΔDRNg1-9 replicated as efficiently as wild-type RCASBP(A) in DF-1 cells and resulted in high levels of RT activity in 293R(A) culture supernatants, demonstrating that virus

particles were now produced in mammalian cells, and thus had an expanded host range for at least one replication cycle. However, the infectivity of the virus released by 293R(A) cells was low compared to the virus released from DF-1 cells. In addition, transfection of RCASBP(A)ΔDRNg1-9 DNA into 293R(A) cells did not initiate an infection that could spread through the culture.

To determine whether altering the envelope proteins could improve the infectivity of particles produced by 293R(A) cells, the subgroup (A) envelope in RCASBP(A)ΔDRNg1-9 was replaced with the avian subgroup (B) envelope and the adapted (M2C) amphotropic MLV envelope (Barsov and Hughes, *J. Virol.* 70:3922-9 1996). The resulting viral clones (RCASBPΔDRNg1-9B and RCASBPΔDRNg1-9M2C) were transfected into DF-1 cells and the infectious virus stocks produced by DF-1 cells transferred to 293R(B) or 293 cells, respectively. The results paralleled those observed with the (A) virus. Although RT activity was detected in the culture supernatants of infected 293R(B) and 293 cells, the virions produced were poorly infectious and did not efficiently transfer the infection to fresh 293R(B) or 293 cells.

EXAMPLE 9

Generation and Expression of RCASBP(A)ΔDRNg1-9gfp

To easily monitor viral infection, green fluorescent protein (GFP, Genbank accession no. U55761) was cloned into the ClaI site of RCASBP(A)ΔDRNg1-9 (EXAMPLE 8) using standard cloning methods (Clontech, Palo Alto, CA, pEGFP-1).

DF-1 and 293R(A) cells infected with RCASBPΔDRNg1-9gfp (FIG. 5B) expressed GFP as detected by fluorescence microscopy. However, the virus was not serially passaged from 293R(A) to 293R(A) cells. The *gfp* gene was also cloned into RCASBPΔDRNg1-9B and 1-9M2C (EXAMPLE 8). The 293R(B) and 293 cells infected with supernatants from DF-1 were bright green, but supernatants from these cells did not efficiently transfer infection (fluorescence) to 293R(B) or 293 cells, respectively. This analysis confirmed that replacing the (A) envelope with a (B) or (M2C) envelope did not markedly enhance the infectivity of virions produced by 293 cells. Therefore, the inability of RCASBPΔDRNg1-9 virus generated by 293 cells to transfer infection to 293 cells is not related to the envelope glycoproteins or is common to all the envelopes tested.

EXAMPLE 10

Long-Term Selection of Viruses with an Altered Host Range

Short-term selection by passage of the chimeric RCASBP(A)ΔDRMLV (EXAMPLE 6) between DF-1 and 293R(A) cells allowed for the selection of mutations in *gag* that increased the release of virus particles by 293R(A) cells (EXAMPLE 8). Therefore, RCASBP(A)ΔDRNg1-9gfp (containing the *gag* mutations resulting from short-term adaptation) was passaged between these cell lines to select for further changes in the viral genome that enhance the infectivity of the particles produced by 293R(A) cells. DF-1 cells were transfected with RCASBP(A)ΔDRNg1-9gfp and the viral supernatants from these cells used to infect 293R(A) cells.

Using FACS sorting, a population of 293R(A) cells enriched for GFP expression was recovered and expanded. A Coulter XL flow cytometer equipped with Coulter software was used to detect and quantify cells expressing GFP. A Becton-Dickinson FACSTAR PLUS flow cytometer was used for experiments in which fluorescent and non-fluorescent cells were sorted and returned to culture. GFP was excited with a 488 nm line from an Argon laser and the GFP signal was collected using a 525/20 bandpass filter. Viable cells were collected using forward and side light scatter.

The vector containing *gfp* was used to determine efficiency of each infection visually and/or quantitated by FACS analysis. Virus released by 293R(A) cells was transferred back to DF-1 cells and, after a few passages of DF-1 cells, a highly infectious virus stock was produced that efficiently infected 293R(A) cells. Since RCASBP(A) viruses grow rapidly on chicken cells, a few viable virus particles can initiate an infection that spreads through the culture. This made it possible to serially passage the RCASBPΔDRNg1-9gfp virus on 293R(A) cells until relatively little fluorescence was observed upon infection of fresh cells, then recover the infectious particles released.

RT activity was detected in the culture supernatant from 293(A) cells, demonstrating that RCASBPΔDRNg1-9gfp was replication competent in mammalian cells. Therefore, RCASBPΔDRNg1-9gfp developed an expanded host range. However, 293R(A) cells infected with viral supernatants from 293R(A) cells produced only a small number of infectious virions able to transfer fluorescence to new 293R(A) cells. The RCASBPΔDRNg1-9gfp virus was continuously shuttled between DF-1 and 293R(A) for over a year. During this long-term adaptation the production of infectious particles by 293R(A) cells improved. The virus can be shuttled between DF-1 and 293R(A) indefinitely, such that the virus will continue to adapt to enhance its ability to replicate in 293R(A) cells.

These experiments yielded a viral stock that can be passed at least twice on 293R(A) cells. After a third passage, little or no RT activity was detectable in the culture supernatants. However, even after three viral passages on 293R(A) cells it was possible to "rescue" the virus on DF-1 cells. The number of productive passages on 293R(A) cells depends on the titer of the original viral supernatant produced by DF-1 cells.

Portions of the genome of the long-term adapted virus were sequenced to identify any changes that had occurred in *gag* or in the 3' UTR. Hirt DNA was prepared and sequenced (see EXAMPLE 4). Two additional point mutations were observed in the MA coding region (FIG. 6 and SEQ ID NO: 13). One mutation was silent at the amino acid level, but the other replaced a Gly with Glu (Table 1). Further mutations were observed in the 3' UTR. The MLV U3 insert and downstream flanking sequence remained intact. However, the first two thirds of *gfp* was deleted as well as the sequence between *env* and ClaI containing a splice acceptor and the coding sequence for the last two amino acids and stop codon of Env. The loss of most of *gfp* (including the region containing the chromaphore) explains the observation that the cell-associated fluorescence faded as the RCASBPΔDRU3FNg1-9gfp virus was passaged.

EXAMPLE 11

Southern Analysis of Infected 293R(A) Cells

Southern blot analysis was performed on genomic DNA from infected 293R(A) cells to determine whether the provirus was intact in these cells (FIG. 7). Genomic DNA was isolated from infected DF-1 cells, from 293R(A) cells infected with viral supernatants from DF-1 cells, and from cells infected after two more passages of the virus from 293R(A) cells to 293R(A) cells. Genomic DNA was extracted using standard techniques (Sambrook *et al.* In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989) or a Qiagen blood and tissue DNA extraction kit according to the manufacturer's instructions.

Genomic DNA (10 µg) was digested overnight with EcoRI. The resulting DNA fragments were separated on a 1% agarose gel and transferred to nitrocellulose. Proviral DNA was detected using ^{32}P labeled fragments of RCASBP(A) DNA. The fragments were generated by digesting the plasmid with PvuI to remove pBR322 sequences, then digesting the gel-isolated viral genome with NcoI. The resulting fragments of the viral genome were labeled in one reaction.

As shown in FIG. 7, many proviruses present in 293R(A) cells infected with viral supernatants produced on 293R(A) cell were extensively deleted. The EcoRI digest of the proviral DNA in these cells gave a consistent pattern of distinct DNA fragments, demonstrating that the deletions are specific.

To identify which portions of the provirus remained, specific fragments of the viral genome were used to probe Southern blots (FIG. 8). The central portion of the genome was lost on viral passage in 293R(A) cells. The proviral DNA in 293R(A) cells contained the upstream LTR, a portion of *gag*, most or all of *env*, and the downstream LTR. The size of the proviral DNA in 293R(A) cells was similar to that of the spliced message that serves as a template for translation of the viral Env protein. This message includes R, U5, U3 and *env*.

In the RCAS vectors, the splice donor site normally used for the production of env mRNA is located 289 nucleotides from the 5' end of the genomic message and just downstream from the start of *gag* (FIG. 9). The *env* splice acceptor is approximately 5 kb downstream near the 3' end of *pol*. Using PCR, a DNA fragment that spanned the deletion found in the proviruses present in 293R(A) genomic DNA was amplified. PCR primers that hybridized to the primer binding site located immediately 3' of the upstream LTR (5' GGTGACCCCGACGTGATAGTT 3', SEQ ID NO: 20) and to a sequence within the gp37 coding region of *env* (5' GGACCCCAAAGCTGCACTTCA 3', SEQ ID NO: 21) were used. PCR was conducted as described in EXAMPLE 4, except the melting and annealing temperatures were raised to 92°C and 61°C, respectively. The resulting DNA fragment was purified and sequenced.

Sequence analysis demonstrated that in the deleted proviruses present in 293R(A) cells, the splice donor at the beginning of *gag* was not used. Splicing occurred instead at the 3' end of the MA coding region (FIG. 9), using a donor sequence not ordinarily recognized by chicken host cells. However, in 293R(A) cells, splicing joins this cryptic donor sequence (located at the end of the MA

coding region) with the splice acceptor site used for the production of the env message (near the end of *pol*). As shown in FIG. 9, short-term adapted RCASBPΔDRNg1-9 genomic RNA (which has six point mutations in *gag*) is spliced at cryptic splice donor site 1 in addition to the splice donor site normally used. Long-adapted RCASBPΔDRNg1-9 genomic RNA (which has eight point mutations in *gag*) is spliced at cryptic splice site 2 in addition to the splice donor site normally used.

Therefore, the *gag* mutations may cause changes in RNA structure that influence RNA recognition by cellular factors. As a result, the same species of retroviral RNA may be differentially spliced in avian and mammalian cells. For example, 293R(A) cells infected with the long-term adapted RCASBPΔDRNg1-9 generate a spliced message not normally found in avian cells. The two viral RNAs typically produced in infected avian cells (full-length genomic RNA and the env spliced message) are also made at some level, because infected 293R(A) cells generate some infectious virions.

EXAMPLE 12

Analysis of Viral RNA Packaged into Virus Particles

As described above, as RCASBPΔDRNg1-9 is passaged on 293R(A) cells, infectivity decreases. Similarly, when virus containing *gfp* was passaged, the percentage of green cells in the newly infected culture declined with each viral passage. Therefore, virus particles assembled in 293R(A) cells (as opposed to avian cells) may package the aberrantly spliced RNA described above along with unspliced RNA.

Culture supernatants from virus-infected DF-1 and 293R(A) cells were centrifuged at low speed (3000 rpm) to remove cells and debris. Virus particles were collected by centrifugation of the clarified supernatants at 35,000 rpm for one hour. Viral RNA was isolated using a Qiagen QIAamp viral RNA mini kit according to the manufacturer's instructions.

Viral RNA was reverse transcribed into DNA in a reaction buffer (25 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM DTT, pH 8.3) containing 1 mM dNTPs, 2 μg random hexamer primers, and 22 units of AMV RT (Avian Myeloblastosis Virus reverse transcriptase). The reaction was incubated for one hour at 42°C. The resulting viral DNA served as the template in PCR amplifications using Perkin Elmer AmpliTaq DNA polymerase. PCR conditions were 30 cycles of 40 seconds at 90°C, 1 minute 20 seconds at 59°C, and 1 minute at 72°C. The PCR primers used to detect unspliced RNA in virus particles were 5' GCGGCAGCCACTCGCGACCCC 3' (SEQ ID NO: 22) and 5' GGCGCCCCCTGTTGGACGGCCCC 3' (SEQ ID NO: 23) and primers 5' GGTGACCCCGACGTGATAGTT 3' (SEQ ID NO: 24) and 5' GGCCTGTACGGTTGGCCCATG 3' (SEQ ID NO: 25) were used and to detect aberrantly spliced RNA in virus particles. The resulting products were separated on a 1% agarose gel and observed using ethidium bromide.

Unspliced RNA was detected in virus particles using primers that amplified a fragment from the region of *gag* located downstream of the cryptic splice site. Unspliced RNA was present in virus particles produced by DF-1 and 293R(A) cells infected with short-term adapted RCASBPΔDRNg1-

9gfp (EXAMPLE 9) and by 293R(A) cells infected with long-term adapted RCASBPΔDRNg1-9gfp (EXAMPLE 10).

To detect aberrantly spliced RNA, primers that annealed on either side of the deletion in proviral DNA were used. Virus particles produced by 293R(A) cells infected with long-term adapted RCASBPΔDRNg1-9gfp virus packaged a spliced message that corresponded in size to the deleted proviruses in infected 293R(A) cells. Virus particles produced by DF-1 and 293R(A) cells infected with non-adapted RCASBPΔDRNg1-9gfp contained a spliced message that was about 400 nucleotides smaller than the spliced message detected in 293R(A) cells infected with long-adapted RCASBPΔDRNg1-9gfp. DNA sequence analysis of the smaller fragment amplified from non-adapted RCASBPΔDRNg1-9gfp virus produced in DF-1 and 293R(A) cells revealed a second cryptic splice donor site located in the upstream portion of the MA coding region.

These results demonstrate that the *gag* mutations influence the recognition/use of crypic splice sites by the host cell machinery. Prior to long-term adaptation RCASBPΔDRNg1-9gfp has six point mutations in *gag* and the virions package a spliced message containing about 160 nucleotides of the MA coding region. The two additional mutations found in long-adapted virus facilitate the use of a cryptic splice donor site at the end of the MA coding region in 293R(A) cells.

Virus serially passaged on 293R(A) cells can be rescued on DF-1 cells because 293R(A) cells produce a few particles containing unspliced RNA. These particles initiate an infection that spreads through the culture of DF-1 cells. The *gag* modifications necessary to increase the production of infectious ASLV particles by 293R(A) cells appear to activate cryptic splice sites that are then used by the host cell splicing machinery. The activation of the cryptic splice sites in viral RNA and the packaging of aberrantly spliced RNAs may be a byproduct of the changes in RNA sequence or structure needed to promote viral replication in 293R(A) cells.

EXAMPLE 13

Altering the Host Range of MLV ampho

As disclosed in the EXAMPLES above, a 96-bp sequence (SEQ ID NO: 1) from the U3 region of the MLV ampho LTR rescues the replication-defective ASLV vector RCASBPΔDR in DF-1 cells. This 96-bp sequence lies in the downstream third of the U3 between a series of repeated enhancer elements and the TATA box at the 3' end of U3. Although the sequence contains a CAAT box, it does not contain any other known transcriptional control elements mapped to the MLV LTR.

To determine the effect of deleting the 96-bp sequence (SEQ ID NO: 1) on MLV ampho replication, the following experiments were conducted. A 100-bp fragment was deleted from the downstream U3 of pRR145 by subcloning a ClaI to NotI fragment containing the downstream LTR into KS (Stratagene, LaJolla, CA). The plasmid was digested with XbaI and BssHII and the ends were made blunt with T4 DNA polymerase and ligated. The deleted ClaI to NotI fragment was moved back into pRR145 to create pRR145ΔdsU3 (FIG. 10).

The same 100-bp fragment was also removed from the upstream LTR. pRR145ΔdsU3 was digested with SalI and religated to remove the downstream half of the genome. The XbaI to BssHII fragment was deleted from the upstream LTR, and the SalI insert was reintroduced to reconstitute the vector. The clone with 100-bp removed from both copies of U3 is pRR145ΔU3 (FIG. 10).

The RCASBP DR sequence was ligated into the downstream LTR of pRR145ΔU3. The ClaI-NotI fragment cloned into KS was digested with XbaI and BssHII. A segment containing the DR region was amplified from MluI to BstuI with 5′ and 3′ primers to introduce XbaI and BssHII sites, respectively, to facilitate cloning. The amplified product was cloned into the downstream LTR in KS as an XbaI to BssHII fragment. The downstream LTR containing the DR was moved from KS into pRR145ΔU3 as a ClaI to NotI fragment, creating pRR145ΔU3dsDR (FIG. 10).

pRR145ΔdsU3 and wild-type pRR145 were transfected into 3T3 and DF-1 cells and the progress of the resulting viral infections followed by measuring RT activity in culture supernatants as described in EXAMPLE 3. Virus production from the deleted clone lagged behind wild-type until about passage five. Although the downstream LTR can be reconstituted by recombination with the intact upstream LTR, the lag in virus production indicates that an intact downstream U3 is required for virus production is required for virus production.

The pRR145ΔU3 vector was transfected into DF-1, 3T3 and 293 cells. Productive infections did not develop in mammalian 3T3 or 293 cells, but replicated to about 30% of wild type levels in DF-1 cells. Therefore, this portion of U3 is important for replication of MLV ampho in mouse and human cells, but is not absolutely required in avian cells. Deletion of this U3 fragment alters (contracts) the host range of MLV, by limiting the number of host cells in which the virus is replication competent.

Immunoblot analysis of cell-free supernatants 24 and 48 hours post-transfection were performed using an MLV anti-CA antibody. Virus particles were detected by Western transfer analysis. Immunoblots were probed with rabbit polyclonal antibodies directed against the gag region of ALV or MoMLV, followed by peroxidase labeled goat anti-rabbit secondary antibodies. Nitrocellulose transfer membranes were washed with TBS-T buffer (20 mM Tris, pH 8.3, 150 mM NaCl, and 0.05% Tween-20) and blocked with 5% milk and 1% normal goat serum in TBS-T buffer. The complex was reacted with a chemiluminescent substrate (Boehringer Mannheim) and the immunoblots exposed to film. No gag proteins were detected in supernatants from 293 cells, but transient expression of gag proteins was detected in supernatants from 3T3 cells.

The vector pRR145ΔU3dsDR did not replicate in 293 or 3T3 cells. The replication of pRR145ΔU3dsDR in DF-1 cells was comparable to pRR145ΔU3. The addition of the DR in either the downstream or the upstream LTR did not improve the replication of the pRR145ΔU3 virus in DF-1 cells. The RT activity measured in supernatants from DF-1 cells infected with pRR145ΔU3 and pRR145ΔU3dsDR never reached the level produced by infection with wild type pRR145. Therefore, the fragment deleted from pRR145ΔU3 enhances the replication of MLV ampho in the mammalian cell lines, 3T3 and 293.

EXAMPLE 14

Production of Sequence Variants

Disclosed herein are HRCE nucleotide and amino acid sequences of that can be used to alter the host range of a retrovirus or retroviral vector. In some embodiments, HRCE sequences can be used to alter the host range of a retrovirus or retroviral vector. In one embodiment, a distinctive functional characteristic of a retrovirus or retroviral vector having an altered host range includes, but is not limited to, the ability of such a retrovirus or retroviral vector to be replication competent in a non-host cell, such that the host range has been expanded, for example, an avian retrovirus which is replication competent in non-host mammalian cells.

In another embodiment, a distinctive functional characteristic of a retrovirus or retroviral vector having an altered host range includes, but is not limited to, the ability of such a retrovirus or retroviral vector to no longer be replication competent in a host cell, such that the host range has been contracted (reduced), for example a mammalian retrovirus which is no longer replication competent in host mammalian cells. The ability of a retrovirus or retroviral vector to be replication competent can readily be determined using the assays disclosed herein, for example the RT assay described in EXAMPLE 2.

Having presented HRCE sequences, for example retroviral LTR, DR, and gag sequences, this disclosure facilitates the creation of nucleic acid molecules derived from those disclosed but which vary in their precise nucleotide sequence from those disclosed. Such variants may be obtained through a combination of standard molecular biology laboratory techniques and the nucleotide sequence information disclosed herein.

HRCE variants, fragments, fusions, and polymorphisms will retain the ability to alter host range. In one example, the host range of an avian retrovirus is altered such that it is replication competent in mammalian cells. In yet another embodiment, the host range of a mammalian retrovirus is altered such that it is replication defective in mammalian cells.

Variants and fragments of a retrovirus or retroviral vector retain at least 70%, 80%, 90%, 95%, 98%, or greater sequence identity to the HRCE sequences disclosed herein, and in particular embodiments at least this much identity to SEQ ID NOS: 1, 2, 3, 11, and 13. Variant and fragment sequences of a HRCE maintain the ability to alter host range. Such activity can be readily determined using the assays disclosed herein.

Variant nucleic acid molecules include those created by standard mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Ch. 15). By the use of such techniques, variants may be created which differ in minor ways from those disclosed. Nucleotide sequences which are derivatives of those disclosed herein and which differ from those disclosed by the deletion, addition or substitution of nucleotides while still encoding a retrovirus which have an altered host range, are comprehended by this disclosure.

Also within the scope of this disclosure are small nucleic acid molecules derived from the disclosed nucleic acid molecules. Such small nucleic acid molecules include oligonucleotides suitable for use as hybridization probes or PCR primers. These small DNA molecules may comprise at least a segment of a HRCE and, for the purposes of PCR, will comprise at least a 20, 30, 40 or 50 contiguous nucleotides of SEQ ID NOS: 1, 2, 3, 11, or 12, or their complementary strands. Longer length nucleotide sequences will provide greater specificity in hybridization or PCR applications than shorter length sequences. Accordingly, superior results may be obtained using longer stretches of consecutive nucleotides.

Nucleotide sequences derived from the disclosed nucleic acid molecules as described above may also be defined as nucleic acid sequences which hybridize under stringent conditions to the nucleic acid sequences disclosed, or fragments thereof. Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (such as Na^+ concentration) of the hybridization buffer determines the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Chapters 9 and 11), herein incorporated by reference. By way of illustration only, a hybridization experiment may be performed by hybridization of a DNA molecule (for example, a variant of *gag*, for example those shown in Table 1) to a target nucleic acid molecule (for example, wild-type *gag*) which has been electrophoresed in an agarose gel and transferred to a nitrocellulose membrane by Southern blotting (Southern, *J. Mol. Biol.* 98:503, 1975), a technique well known in the art and described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Specific hybridization refers to the binding, duplexing, or hybridizing of a molecule only or substantially only to a particular nucleotide sequence when that sequence is present in a complex mixture (e.g. total cellular DNA or RNA). Specific hybridization may also occur under conditions of varying stringency.

Hybridization with a target probe labeled with [^{32}P]-dCTP is generally carried out in a solution of high ionic strength such as 6xSSC at a temperature that is about 5-25°C below the melting temperature, T_m. For such Southern hybridization experiments where the target DNA molecule on the Southern blot contains 10 ng of DNA or more, hybridization is typically carried out for 6-8 hours using 1-2 ng/ml radiolabeled probe (of specific activity equal to 10^9 CPM/μg or greater). Following hybridization, the nitrocellulose filter is washed to remove background hybridization. The washing conditions should be as stringent as possible to remove background hybridization but to retain a specific hybridization signal.

The T_m of such a hybrid molecule may be estimated from the following equation (Bolton and McCarthy, *Proc. Natl. Acad. Sci. USA* 48:1390, 1962): $T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - 0.63(\%\ formamide) - (600/l)$; where l = the length of the hybrid in base pairs. This

equation is valid for concentrations of Na^+ in the range of 0.01 M to 0.4 M, and it is less accurate for calculations of T_m in solutions of higher [Na^+]. The equation is also primarily valid for DNAs whose G+C content is in the range of 30% to 75%, and it applies to hybrids greater than 100 nucleotides in length (the behavior of oligonucleotide probes is described in Ch. 11 of Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). In the present disclosure, the equation can be applied to a probe of about 96 nucleotides in length.

Thus, by way of example, for a 150 base pair DNA probe derived from the open reading frame of *gag* (with a hypothetical %GC = 45%), a calculation of hybridization conditions required to give particular stringencies may be made. It is assumed that the filter is washed in 0.3 xSSC solution following hybridization, thereby: [Na^+] = 0.045 M; %GC = 45%; Formamide concentration = 0; l = 150 base pairs; T_m=81.5 – 16.6($\log_{10}$[Na^+]) + (0.41 x 45) - (600/150); and so T_m = 74.4°C.

The T_m of double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner *et al.*, *J. Mol. Biol.* 81:123, 1973). Therefore, for this given example, washing the filter in 0.3 xSSC at 59.4-64.4°C will produce a stringency of hybridization equivalent to 90%; that is, DNA molecules with more than 10% sequence variation relative to the target cDNA (for example *gag*) will not hybridize. Alternatively, washing the hybridized filter in 0.3 xSSC at a temperature of 65.4-68.4°C will yield a high hybridization stringency of 94%; that is, nucleic acid molecules with more than 6% sequence variation relative to the target nucleic acid molecule will not hybridize. The above example is given entirely by way of theoretical illustration. One skilled in the art will appreciate that other hybridization techniques may be utilized and that variations in experimental conditions will necessitate alternative calculations for stringency.

Examples of stringent conditions are those under which DNA molecules with more than 25%, 15%, 10%, 6% or 2% sequence variation (also termed "mismatch") will not hybridize. Stringent conditions are sequence dependent and are different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions include a temperature approximately 0-20°C below the calculated T_m, for example no more than about 5°C lower than the thermal melting point T_m, at a defined ionic strength and pH. An example of stringent conditions is a salt concentration of at least about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and a temperature of at least about 30°C for short probes (e.g. 10 to 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. For example, conditions of 5X SSPE (750 mM NaCl, 50 mM Na Phosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C are suitable for allele-specific probe hybridizations. In another embodiment, hybridization conditions include wash conditions at a temperature of, for example, 48°C, 58°C, 62°C or 68°C, with between 2X and 0.1X SSC and about 0.5% SDS, for instance, 62°C with 0.1X SSC and 0.5% SDS. Exact experimental hybridization conditions are determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to a probe of interest and then washed under conditions of different stringencies.

A perfectly matched probe has a sequence perfectly complementary to a particular target sequence. The test probe is typically perfectly complementary to a portion (subsequence) of the target sequence. The term "mismatch probe" refers to probes whose sequence is deliberately selected not to be perfectly complementary to a particular target sequence.

One skilled in the art will recognize that the mutagenesis techniques described above may be used not only to produce variant nucleic acid molecules, but will also facilitate the production of RNA which differ in certain structural aspects from a HRCE, yet which maintain the essential functional characteristic of the HRCE.

While the site for introducing a nucleic acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target region and the variants screened for optimal activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence as described above are well known.

Nucleic acid substitutions include single residues; for example 1, 2, 3, 4, 5, 10 or more substitutions; insertions of about from 1 to 10 residues; and deletions from about from 1 to 30 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct.

Such variants can be readily selected for additional testing by performing an assay (such as those described in EXAMPLE 2) to determine if the variant HRCE can still alter the host range of a retrovirus or retroviral vector.

EXAMPLE 15

Pharmaceutical Compositions and Modes of Administration

Various delivery systems for administering the retroviruses and retroviral vectors having an altered host range disclosed herein are known, and include e.g., encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (see Wu and Wu, *J. Biol. Chem.* 1987, 262:4429-32), and construction of therapeutic nucleic acids as part of a retroviral or other vector. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, the pharmaceutical compositions may be introduced into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Administration can be systemic or local. The retroviral vectors of the disclosure can be administered together with other biologically active agents.

In one embodiment, it may be desirable to administer the pharmaceutical compositions disclosed herein locally to the area in need of treatment, for example, by local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, through a catheter, by a suppository or an implant, such as a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. In one embodiment, administration can be by direct administration at a site where gene therapy is desired.

The present disclosure also provides pharmaceutical compositions which include a therapeutically effective amount of a retrovirus or retroviral vector having an altered host range disclosed herein, alone or with a pharmaceutically acceptable carrier.

Delivery systems

The pharmaceutically acceptable carriers useful herein are conventional. *Remington's Pharmaceutical Sciences*, by Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the retroviral vectors of the disclosure. In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, sesame oil, glycerol, ethanol, combinations thereof, or the like, as a vehicle. The carrier and composition can be sterile, and the formulation suits the mode of administration. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, sodium saccharine, cellulose, magnesium carbonate, or magnesium stearate. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

The amount of retrovirus or retroviral vector that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The disclosure also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. Instructions for use of the composition can also be included.

The pharmaceutical compositions or methods of treatment may be administered in combination with other therapeutic treatments, such as other antineoplastic or antitumorigenic therapies.

The disclosure provides compositions of the retroviruses and retroviral vectors disclosed herein, for example a composition that is comprised of at least 90% of a retrovirus or retroviral vector in the composition. Such compositions are useful as therapeutic agents when constituted as pharmaceutical compositions with the appropriate carriers or diluents.

Embodiments of the disclosure comprising medicaments can be prepared with conventional pharmaceutically acceptable carriers, adjuvants and counterions as would be known to those of skill in the art.

Having illustrated and described methods for generating and selecting for retroviruses and retroviral vectors having an altered host range, the viruses obtained using this method, and the use of such viruses, it should be apparent to one skilled in the art that the disclosure can be modified in arrangement and detail without departing from such principles. In view of the many possible embodiments to which the principles of our disclosure may be applied, it should be recognized that the illustrated embodiments are only particular examples of the disclosure and should not be taken as a limitation on the scope of the disclosure. Rather, the scope of the disclosure is in accord with the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A method for identifying a host range control element (HRCE) in a first retroviral vector, wherein the first retroviral vector is replication competent in a first host cell, comprising:

functionally deleting a HRCE in a second retroviral vector, wherein the second retroviral vector is replication competent in a second host cell;

incorporating a nucleic acid sequence from the first retroviral vector into the second retroviral vector, thereby generating a third retroviral vector;

transfecting the second host cell with the third retroviral vector;

assaying replication of the third retroviral vector in the second host cell; and

determining if the third retroviral vector is replication competent in the transfected second host cell, wherein replication competency identifies the HRCE of the first retroviral vector.

2. The method of claim 1, further comprising:

functionally deleting the HRCE of the first retroviral vector, thereby generating a HRCE-deleted first retroviral vector;

transfecting the first host cell with the HRCE-deleted first retroviral vector;

assaying the transfected first host cell for replication competency of the HRCE-deleted retroviral vector; and

determining if the HRCE-deleted retroviral vector was replication competent in the transfected first host cell, thereby determining if deletion of the HRCE altered the host range of the first retroviral vector.

3. The method of claim 1, wherein the first and second retroviral vectors are simple retroviral vectors.

4. The method of claim 1, wherein the third retroviral vector is a chimeric retroviral vector.

5. The method of claim 1, wherein assaying the transfected second host cell for replication competency of the third retroviral vector comprises assaying for reverse transcriptase (RT) activity.

6. The method of claim 1, wherein the first retroviral vector is a mammalian retroviral vector.

7. The method of claim 6, wherein the mammalian retroviral vector is a murine leukemia virus (MLV) ampho and the first host cell is a mammalian or avian cell.

8. The method of claim 1, wherein the second retroviral vector is an avian retroviral vector and the second host cell is an avian cell.

9. The method of claim 8, wherein the avian retroviral vector is an avian sarcoma leukosis virus (ASLV) and the second host cell is DF-1.

10. The method of claim 1, wherein the functionally deleted HRCE in the second retroviral vector is a direct repeat (DR), a constitutive transport element (CTE), a long terminal repeat (LTR), or portion thereof.

11. The method of claim 10, wherein the second retroviral vector is ASLV and the functionally deleted HRCE is a DR or portion thereof.

12. The method of claim 10, wherein the second retroviral vector is MLV ampho and the functionally deleted HRCE is a LTR or portion thereof.

13. The method of claim 1, wherein the nucleic acid sequence from the first retroviral vector is included in a heterogeneous mixture of nucleic acid sequences from the first retroviral vector.

14. The method of claim 13, wherein the heterogeneous mixture of nucleic acid sequences from the first retrovirus comprises a library of the first retroviral vector.

15. The method of claim 1, wherein the nucleic acid sequence comprises at least 10 nucleotides.

16. The method of claim 15, wherein the nucleic acid sequence comprises at least 100 nucleotides.

17. The method of claim 1, wherein the nucleic acid sequence is not an envelope sequence or fragment thereof.

18. The method of claim 1, further comprising identifying an additional nucleic acid sequence in the second retroviral vector that affects the host range of the second retroviral vector.

19. The method of claim 18, wherein the nucleic acid sequence from the first retroviral vector is an HRCE.

20. The method of claim 18, wherein identification of the additional nucleic acid sequence comprises;

(a) collecting culture supernatant from the transfected second host cell containing a third retroviral vector virion;

(b) using the supernatant to transfect a third host cell; and

(c) collecting a culture supernatant from the transfected third host cell containing the third retroviral vector virion.

21. The method of claim 20, further comprising, repeating steps (a)-(c) until a third retroviral vector population is obtained that is replication competent in the third host cell.

22. The method of claim 20, wherein the third host cell and the first host cell are from the same species.

23. The method of claim 20, wherein the second host cell is an avian cell.

24. The method of claim 23, wherein the avian cell is a chicken cell.

25. The method of claim 20, wherein the first host cell is a mammalian cell.

26. The method of claim 20, wherein the first retroviral vector is an avian or mammalian retroviral vector.

27. The method of claim 18, wherein the additional nucleic acid sequence contains mutations in *gag*.

28. A method for identifying a HRCE in a mammalian retroviral vector, comprising:

functionally deleting a DR in an avian retroviral vector, wherein the avian retroviral vector is replication competent in an avian host cell;

incorporating a nucleic acid sequence from the mammalian retroviral vector into the avian retroviral vector, thereby generating a chimeric retroviral vector;

transfecting the avian host cell with the chimeric retroviral vector;

assaying the transfected avian host cell for replication competency of the chimeric retroviral vector; and

determining if the chimeric retroviral vector is replication competent in the transfected avian host cell, thereby identifying the HRCE of the mammalian retroviral vector.

29. A method for generating a retroviral vector having an altered host range, comprising functionally deleting a HRCE in a first retroviral vector, thereby generating an HRCE-deleted retroviral vector, wherein the HRCE is not an envelope sequence or portion thereof.

30. The method of claim 29, further comprising replacing the functionally-deleted HRCE with an HRCE from a second retroviral vector, thereby generating a chimeric retroviral vector.

31. The method of claim 29, wherein the first retroviral vector is a mammalian retroviral vector.

32. The method of claim 31 wherein the mammalian retroviral vector is an MLV ampho retroviral vector.

33. The method of claim 30, wherein the second retroviral vector and the first retroviral vector have a different host range.

34. The method of claim 31, wherein the functionally-deleted HRCE comprises a portion of a U3 region of an LTR.

35. The method of claim 34, wherein the U3 region comprises at least 15 but no more than 614 contiguous nucleotides of the LTR wherein the HRCE-deleted retroviral vector has an altered host range.

36. The method of claim 35, wherein the U3 region comprises at least 96 but no more than 614 contiguous nucleotides of the LTR, wherein the HRCE-deleted retroviral vector has an altered host range.

37. The method of claim 31, wherein the functionally-deleted HRCE comprises at least 50 nucleotides having at least 70% identity to SEQ ID NO: 1, 2, or 3, wherein the HRCE-deleted retroviral vector has an altered host range.

38. The method of claim 37, wherein the functionally-deleted HRCE comprises at least 50 nucleotides having at least 90% identity with SEQ ID NO: 1, 2,or 3, wherein the HRCE-deleted retroviral vector has an altered host range.

39. The method of claim 38, wherein the functionally-deleted HRCE comprises SEQ ID NO: 1 and the HRCE-deleted retroviral vector has an altered host range.

40. The method of claim 31, wherein the functionally-deleted HRCE comprises at least 50 nucleotides that hybridize with a complement of a SEQ ID NO:1, 2 or 3, wherein hybridization conditions comprise wash conditions of 0.1 X SSC, 0.5% SDS at 62°C, and wherein the HRCE-deleted retroviral vector has an altered host range.

41. The method of claim 30, wherein the functionally deleted HRCE is a DR or portion thereof.

42. The method of claim 30, wherein the second HRCE from the second retrovirus comprises a sequence having at least 70% sequence identity to SEQ ID NO: 1, 2 or 3, wherein the chimeric retroviral vector has an altered host range.

43. The method of claim 42, wherein the second HRCE from the second retrovirus comprises SEQ ID NO: 1, 2, or 3.

44. The method of claim 30, wherein the functionally-deleted HRCE is an ASLV DR and the second HRCE is a sequence having at least 70% sequence identity to SEQ ID NO: 11 or 13, wherein the chimeric retroviral vector has an altered host range.

45. The method of claim 30, wherein the first retroviral vector is an ASLV, the HRCE from ASLV is a DR, and the HRCE from the second retroviral vector is SEQ ID NO: 1 wherein the ASLV has an altered host range.

46. A method for generating an MLV ampho retroviral vector having an altered host range, comprising functionally deleting an LTR or portion thereof from the MLV ampho retroviral vector, wherein the functionally deleted LTR or portion thereof comprises a sequence having 70% sequence identity SEQ ID NO: 1 or 3.

47. The method of claim 46, wherein the LTR comprises SEQ ID NO: 1 or 3.

48. The method of claim 29, further comprising:

(a) incorporating a nucleic acid sequence from a second retroviral vector having a second host cell range into the first retroviral vector, thereby generating a third retroviral vector;

(b) transfecting a third host cell with the third retroviral vector;

(c) collecting a cell free supernatant containing retroviral particles from the transfected third host cell;

(d) using the cell free supernatant containing the retroviral particles to infect a fourth host cell; and

(e) collecting a cell free supernatant containing retroviral particles from the fourth host cell.

49. The method of claim 48, further comprising repeating (b)-(e) to select a population of retroviral particles that is replication competent in the first host cell.

50. The method of claim 48, wherein the third host cell and the first host cell are from the same species and the fourth host cell and the second host cell are from the same species.

51. The method of claim 48, wherein the third host cell and the second host cell are from the same species and the fourth host cell and the first host cell are from the same species.

52. A method for generating an ASLV that is replication competent in a mammalian cell comprising:

(a) functionally deleting a DR in the ASLV;

(b) incorporating a nucleic acid sequence from a mammalian retroviral vector, thereby generating a chimeric retroviral vector;

(c) transfecting an avian cell with the chimeric retroviral vector;

(d) collecting a cell free supernatant containing chimeric retroviral particles from the transfected avian cell;

(e) using the cell free supernatant containing the chimeric retroviral particles to infect a mammalian cell;

(f) collecting a cell free supernatant containing chimeric retroviral particles from the mammalian cell; and

(g) repeating steps (c)-(f) to select a chimeric retroviral vector that is replication competent in the mammalian cell.

53. The method of claim 52, wherein the mammalian retroviral vector is an MLV ampho.

54. The method of claim 53, wherein the nucleic acid sequence is an MLV ampho HRCE.

55. A retroviral vector having an altered host range obtained using the method of claim 29 or 30.

56. The retroviral vector of claim 56, wherein the retroviral vector is no longer replication competent in at least one native cell type.

57. The retroviral vector of claim 55, wherein the retroviral vector is incorporated in a viral particle.

58. The retroviral vector of claim 55, wherein the retroviral vector is not replication competent in a mammalian cell.

59. A cell infected with the retroviral vector of claim 55.

60. The host cell of claim 59, wherein the cell is a mammalian or avian cell.

61. The host cell of claim 59, wherein the cell is a mammalian cell.

62. The host cell of claim 61, wherein the mammalian cell is a human cell.

63. A non-human mammalian cell infected with the retroviral vector of claim 55, wherein the retroviral vector is integrated into the genome of the non-human mammalian cell.

64. A method for transforming a cell, comprising:

contacting the cell with a retrovirus comprising a retroviral vector of claim 55, wherein the retroviral vector comprises a transgene; and

wherein the contact results in transformation of the cell.

65. The method of claim 64, wherein the retroviral vector is replication defective.

66. The method of claim 64, wherein the retroviral vector further comprises a promoter operably linked to the transgene.

67. The method of claim 64, wherein the introduction is *in vitro* or *in vivo*.

68. The method of claim 64, wherein the retroviral vector is integrated into a chromosome of the cell.

69. The method of claim 64, wherein the transgene is a marker polypeptide or a therapeutic polypeptide.

70. The method of claim 64, wherein no other viral vector is introduced into the cell.

71. A method for preventing or treating disorder in a subject, comprising introducing into a cell of the subject an therapeutically effective amount of the retroviral vector of claim 55, wherein the vector comprises a transgene, wherein the vector is replication defective, wherein the introduction results in the stable genetic transformation of the cell and expression of the transgene, and wherein the expression of the transgene results in alleviating a symptom of the disorder or preventing the disorder.

72. The method of claim 71, wherein the expression vector is introduced into the subject's cells *ex vivo* and the cells are then reintroduced into the subject.

73. A method for treating a subject, comprising contacting a cell of the subject with an therapeutically effective amount of the retroviral vector of claim 55, wherein the retroviral vector is replication defective and wherein the retroviral vector comprises a transgene, wherein the contact results in the retroviral vector integrating into a chromosome of the cell thereby expressing the transgene in the cell, wherein the cell is not contacted with any other virus, and the expression of the transgene treats the subject.

74. The method of claim 73, wherein the transgene is a therapeutic polypeptide or an antisense sequence.

75. A pharmaceutical composition, comprising:

the retroviral vector of claim 55, wherein the vector comprises a nucleic acid sequence encoding a transgene; and

a pharmaceutically acceptable carrier.

76. The use of SEQ ID NO: 1, 2, 3, 11 or 13 to alter a host range of a retroviral vector.

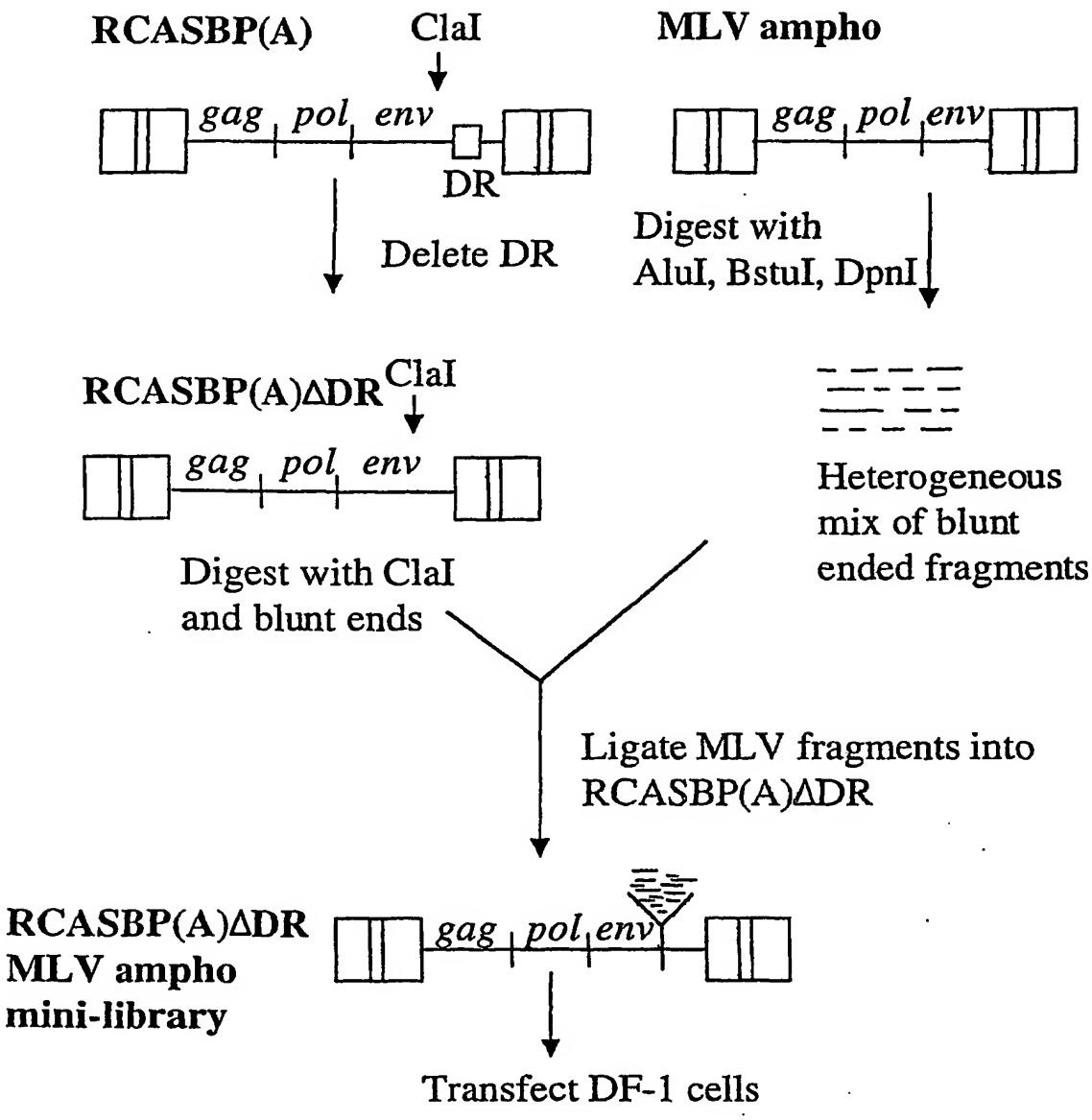

RCASBP(A)
ClaI
MLV ampho
gag pol env
DR
Delete DR
Digest with
AluI, BstuI, DpnI
RCASBP(A)ΔDR
ClaI
gag pol env
Heterogeneous
mix of blunt
ended fragments
Digest with ClaI
and blunt ends
Ligate MLV fragments into
RCASBP(A)ΔDR
RCASBP(A)ΔDR
MLV ampho
mini-library
gag pol env
Transfect DF-1 cells

FIG. 1

FIG. 2

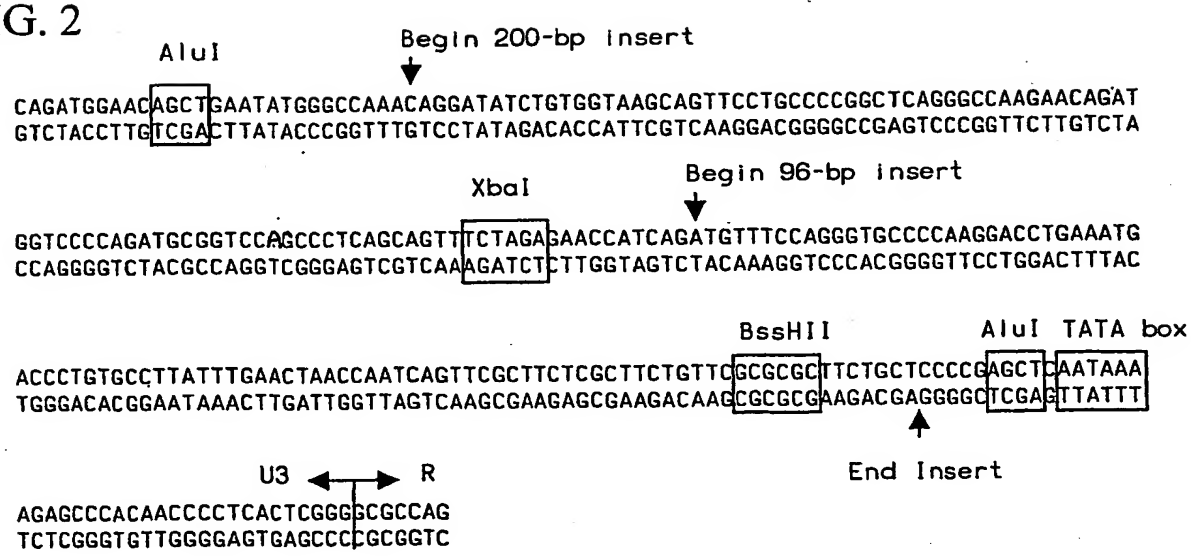


FIG. 3

96-bp insert remaining after adaptation

ATGTTTCCAGGGTGCCCCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGT
TACAAAGGTCCCACGGGGTTCCTGGACTTTACTGGGACAACGGAATAAACTTGATTGGTTAGTCAAGCGAAGAGCGAAGAC

Downstream flanking sequence

TCGCGCGCTTCTGCTGCTGCTTACCGCGGGTATCCTGTTTGGCCCATATTCAG
AGCGCGCGAAGACGA

MA start sd 1
ATGGAAGCCGTCATTAAGGTGATTTCGTCCGCGTGTAAAACCTATTGCGGGA
mutations T

AAATCTCTCCTTCTAAGAAGGAAATAGGGGCCATGTTGTCCCTGTTACAAAA

2
GGAAGGGTTGCTTATGTCTCCCTCAGATTTATATTCTCCGGGGTCCTGGGAT
C

3 cryptic sd
CCATCACTGCGGCGCTCTCCCAGCGGGCAATGGTACTTGGAAAATCGGGAG
A

4
AGTTAAAAACCTGGGGATTGGTTTTGGGGGCATTGAAGGCGGCTCGAGAGGA
C

CAGGTTACATCTGAGCAAGCAAAGTTTTGGTTGGGATTAGGGGGAGGGAGGG

TCTCTCCCCCAGGTCCGGAGTGCATCGAGAAACCAGCTACGGAGCGGCGAAT

5
CGACAAAGGGGAGGAGGTGGGAGAAACAACTGTGCAGCGAGATGCGAAGAT
A

GGCGCCAGAGGAAGCGGCCACACCTAAAACCGTTGGCACATCCTGCTATCAT

TGCGGAACAGCTGTTGGCTGCAATTGCGCCACCGCCACAGCCTCGGCCCCTC

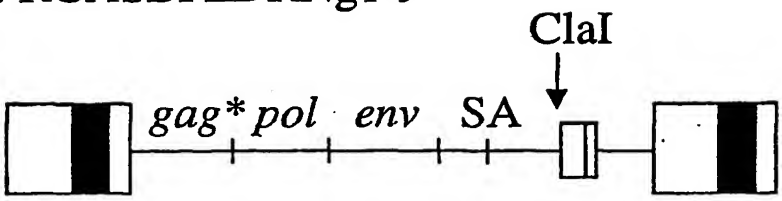
CTCCCCCTTATGTGGGGAGTGGTTTGTATCCTTCCCTGGCGGGGGTGGGAGA
end MA

GCAGCAGGGCCAGGGAGATAACACGTCTCGGGGGGCGGAGCAGCCAAGGGA

6
GGAGCCAGGGCACGCGGGTCAGGCCCCTGGGCCGGCCCTGACTGACTGGGC
T

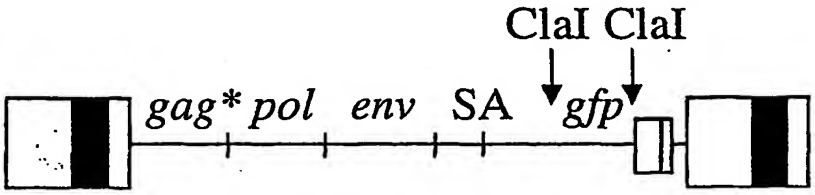
FIG. 4

FIG. 5 A. RCASBPΔDRNg1-9



96-bp MLV U3 insert
and flanking sequence

FIG. 5 B. RCASBPΔDRNg1-9gfp



96-bp MLV U3 insert
and flanking sequence

*gag** contains six point mutations

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ATGGAAGCCGTCATTAAGGTGATTTCGTCCGCGTGTAAAACCTATTGCGGGA
mutations T

AAATCTCTCCTTCTAAGAAGGAAATAGGGGCCATGTTGTCCCTGTTACAAAA

2
GGAAGGGTTGCTTATGTCTCCCTCAGATTTATATTCTCCGGGGTCCTGGGAT
C

3 cryptic sd
CCATCACTGCGGCGCTCTCCCAGCGGGCAATGGTACTTGGAAAATCGGGAG
A

4
AGTTAAAAACCTGGGGATTGGTTTTGGGGGCATTGAAGGCGGCTCGAGAGGA
C

7
CAGGTTACATCTGAGCAAGCAAAGTTTTGGTTGGGATTAGGGGGAGGGAGGG
A

TCTCTCCCCCAGGTCCGGAGTGCATCGAGAAACCAGCTACGGAGCGGCGAAT

5 8
CGACAAAGGGGAGGAGGTGGGAGAAACAACTGTGCAGCGAGATGCGAAGAT
A A

GGCGCCAGAGGAAGCGGCCACACCTAAAACCGTTGGCACATCCTGCTATCAT

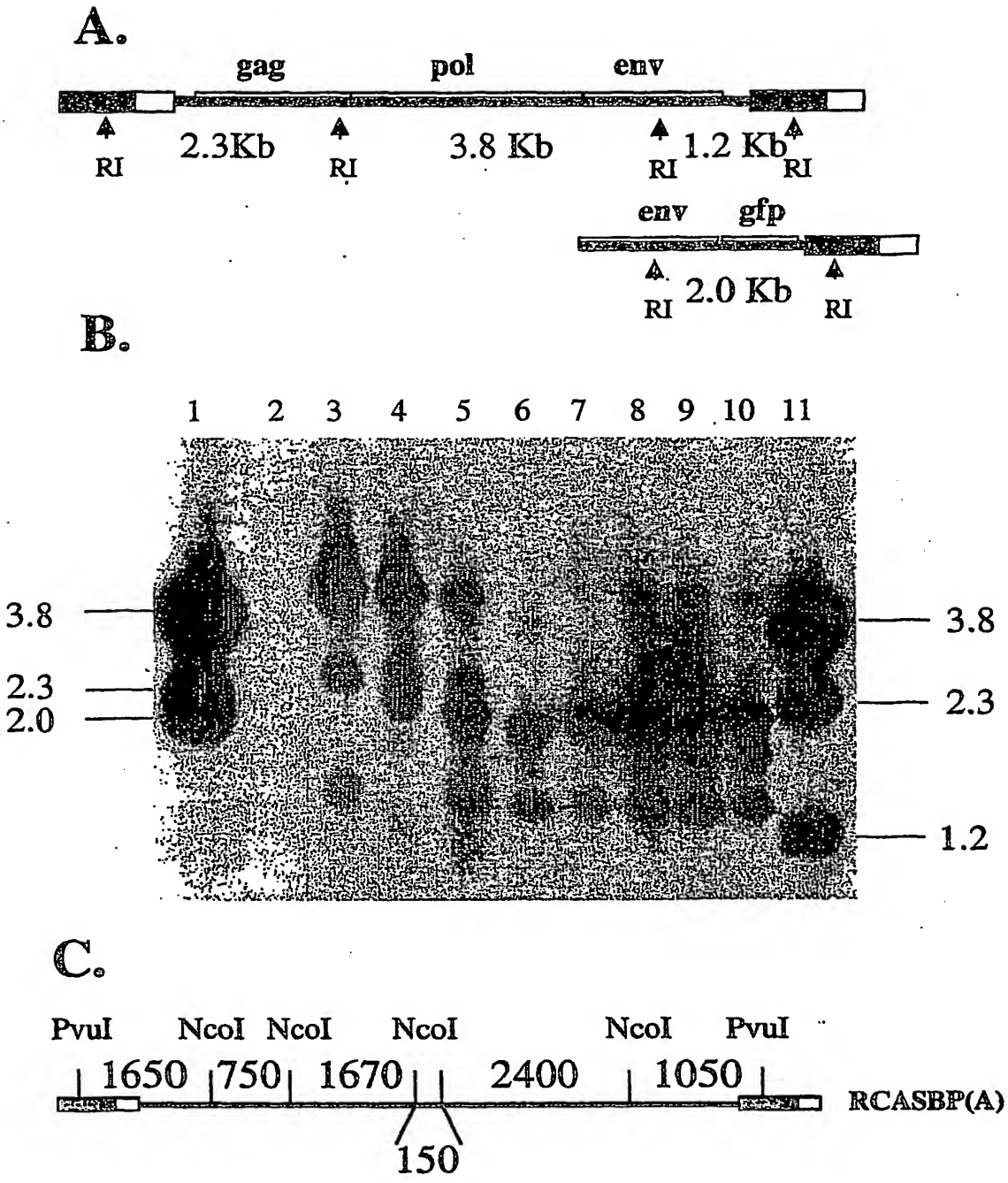
TGCGGAACAGCTGTTGGCTGCAATTGCGCCACCGCCACAGCCTCGGCCCCTC

cryptic sd
CTCCCCCTTATGTGGGGAGTGGTTTGTATCCTTCCCTGGCGGGGGTGGGAGA
end MA

GCAGCAGGGCCAGGGAGATAACACGTCTCGGGGGGCGGAGCAGCCAAGGGA

6
GGAGCCAGGGCACGCGGGTCAGGCCCCTGGGCCGGCCCTGACTGACTGGGC
T

FIG. 6



Combined restriction fragments used as probe

FIG. 7

FIG. 8A

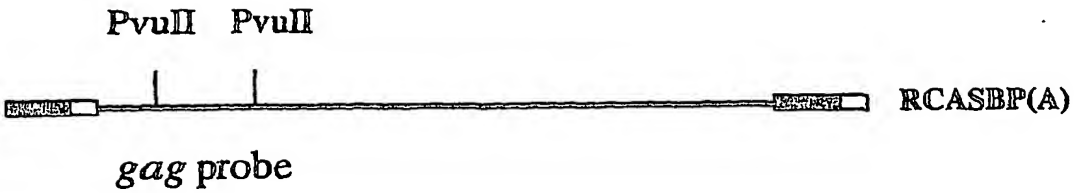


FIG. 8B

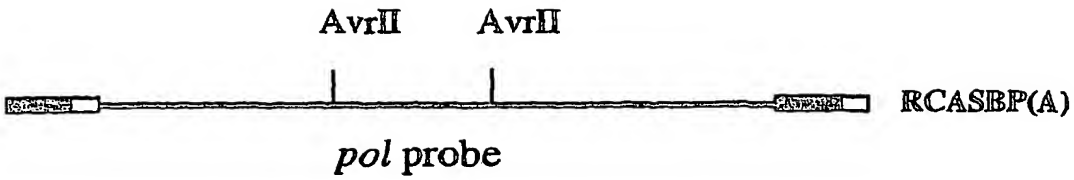
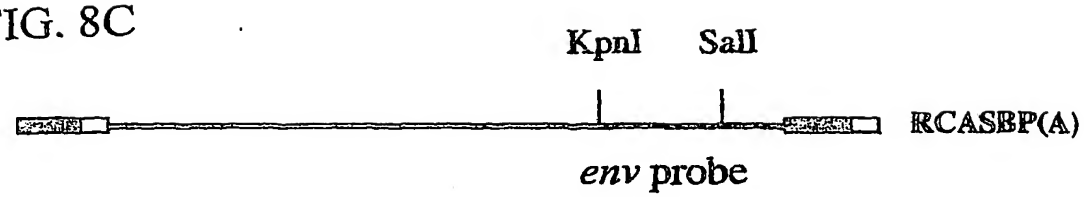


FIG. 8C



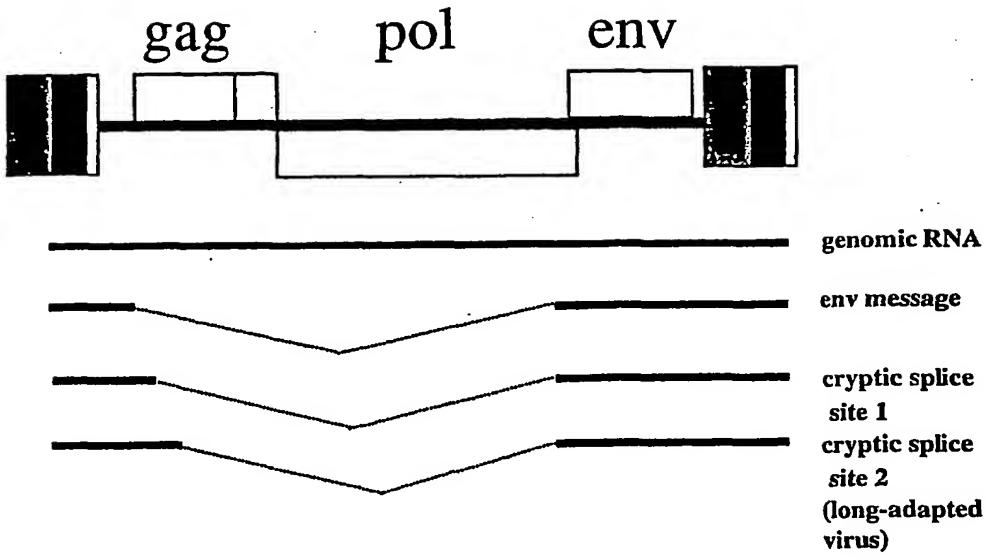
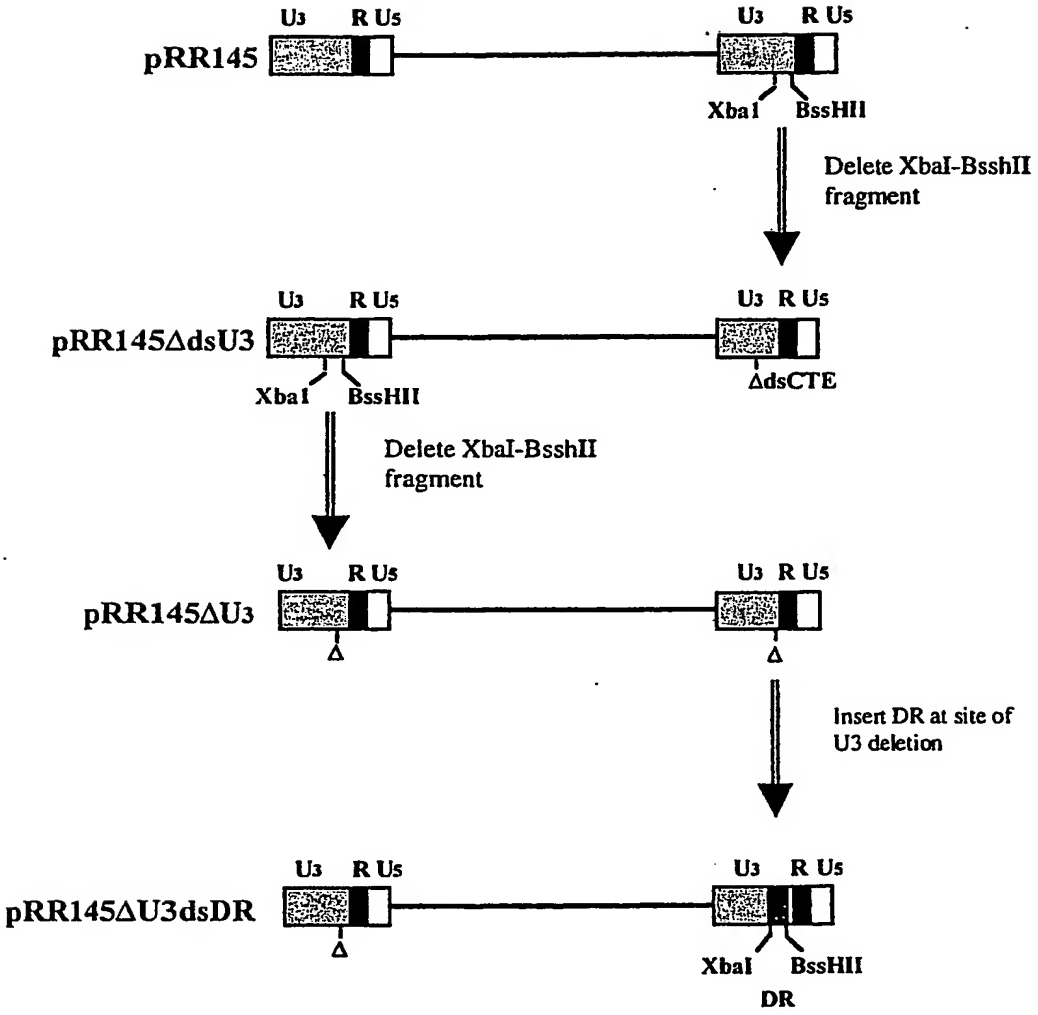


FIG. 9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U3 R U5
U3 R U5
XbaI
BssHII
Delete XbaI-BsshII fragment
pRR145ΔdsU3
U3 R U5
U3 R U5
XbaI
BssHII
ΔdsCTE
Delete XbaI-BsshII fragment
pRR145ΔU3
U3 R U5
U3 R U5
Δ
Δ
Insert DR at site of U3 deletion
pRR145ΔU3dsDR
U3 R U5
U3 R U5
Δ
XbaI
BssHII
DR

FIG. 10

SEQUENCE LISTING

<110> The Government of the United States

<120> Methods to Control the Host Range of Retroviral Vectors

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<150> 60/257,821
<151> 2000-12-22

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<170> PatentIn Ver. 2.1

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ttcgcgcgct tctgct 196

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sequence after short-term selection

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aatcgacaaa ggggaggaag tgggagaaac aactgtgcag cgagatgcga agatggcgcc 420
agaggaagcg gccacaccta aaaccgttgg cacatcctgc tatcattgcg gaacagctgt 480
tggctgcaat tgcgccaccg ccacagcctc ggcccctcct ccccccttatg tggggagtgg 540
tttgtatcct tccctggcgg gggtgggaga gcagcagggc cagggagata acacgtctcg 600
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gactgactgg gc 672

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<223> Description of Artificial Sequence: PCR Primer

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ggcctgtacg gttggcccat g 21

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FERRIS, Andrea, L.** [US/US]; 1570 Dockside Drive, Frederick, MD 21701 (US). **HUGHES, Stephen, H.** [US/US]; 13405 Loy Wolfe Road, Smithsburg, MD 21783 (US).

(74) Agent: **NOONAN, William, D.**; Klarquist, Sparkman, LLP, Suite 1600 - One World Trade Center, 121 S.W. Salmon Street, Portland, OR 97204 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
13 March 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/056668 A3

(54) Title: METHODS TO CONTROL THE HOST RANGE OF RETROVIRAL VECTORS

(57) Abstract: A method for generating and selecting for retroviral vectors having an altered host range, for example an expanded or reduced host range, is disclosed. The method includes manipulating host range control element(s) in a retroviral vector. Methods of using the retroviral vectors are also disclosed.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/50284

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 12339 A (CONKLIN DOUGLAS S ;BEACH DAVID H (US); SUN PEIQUING (US); COLD SPR) 26 March 1998 (1998-03-26) abstract page 3, line 8 -page 4, line 12 ---	1-6, 13-28
A	US 6 096 534 A (BARSOV EUGENE ET AL) 1 August 2000 (2000-08-01) the whole document ---	1-32, 46-74
A	PERKUS M E ET AL: "VACCINIA VIRUS HOST RANGE GENES" VIROLOGY, RAVEN PRESS, NEW YORK, NY, US, vol. 179, 1990, pages 276-286, XP000255782 ISSN: 0042-6822 abstract figure 1 ---	1-6

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 December 2002	16/12/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BARSOV E ET AL: "GENE TRANSFER INTO MAMMALIAN CELLS BY A ROUS SARCOMA VIRUS -BASED RETROVIRAL VECTOR WITH THE HOST RANGE OF THE AMPHOTROPIC MURINE LEUKEMIA VIRUS" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 70, no. 6, 1 June 1996 (1996-06-01), pages 3922-3929, XP002011559 ISSN: 0022-538X cited in the application	1-4
A	the whole document	5-32
A	YEE J-K ET AL: "GENERATION OF HIGH-TITER PSEUDOTYPED RETROVIRAL VECTORS WITH VERY BROAD HOST RANGE" METHODS IN CELL BIOLOGY, LONDON, GB, vol. 43, 1994, pages 99-112, XP002930286 the whole document	1-32
A	MORGAN R A ET AL: "ANALYSIS OF THE FUNCTIONAL AND HOST RANGE-DETERMINING REGIONS OF THE MURINE ECOTROPIC AND AMPHOTROPIC RETROVIRUS ENVELOPE PROTEINS" JOURNAL OF VIROLOGY, NEW YORK, US, US, vol. 67, no. 8, August 1993 (1993-08), pages 4712-4721, XP001062470 ISSN: 0022-538X abstract figures 1,4	1-32
A	JIANYUN DONG ET AL: "A CHIMERIC AVIAN RETROVIRUS CONTAINING THE INFLUENZA VIRUS HEMAGGLUTININ GENE HAS AN EXPANDED HOST RANGE" JOURNAL OF GENERAL VIROLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, READING, GB, vol. 66, no. 12, 1 December 1992 (1992-12-01), pages 7374-7382, XP000572226 ISSN: 0022-1317 abstract figures 1,2	1-6
A	LUCHER L A: "ABORTIVE ADENOVIRUS INFECTION AND HOST RANGE DETERMINANTS" CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, SPRINGER, BERLIN,, DE, vol. 199, 1995, pages 119-152, XP002926241 ISSN: 0070-217X paragraphs '0001!,'0005!	1-32

-/--

INTERNATIONAL SEARCH REPORT

Inter[illegible]nal A[illegible]ion No
PCT/US 01/50284

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BROUGH D ET AL: "RESTRICTED CHANGES IN THE ADENOVIRUS DNA-BINDING, PROTEIN THAT LEAD TO EXTENDED HOST RANGE OR TEMPERATURE-SENSITIVE PHENOTYPES" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 55, no. 1, 1 July 1985 (1985-07-01), pages 206-212, XP002012304 ISSN: 0022-538X abstract	1-6
A	BEATTIE E ET AL: "HOST-RANGE RESTRICTION OF VACCINIA VIRUS E3L-SPECIFIC DELETION MUTANTS" VIRUS GENES, KLUWER ACADEMIC PUBLISHERS, BOSTON, US, vol. 12, no. 1, 1996, pages 89-94, XP000990662 ISSN: 0920-8569 abstract	1-6
A	ADAMS R M ET AL: "INFECTION BY RETROVIRAL VECTORS OUTSIDE OF THEIR HOST RANGE IN THE PRESENCE OF REPLICATION-DEFECTIVE ADENOVIRUS" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 69, no. 3, 1 March 1995 (1995-03-01), pages 1887-1894, XP002004830 ISSN: 0022-538X abstract	1-6

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/50284

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 71-74 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/50284

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9812339	A	26-03-1998	US	6025192 A	15-02-2000
			US	6255071 B1	03-07-2001
			AU	738156 B2	13-09-2001
			AU	4659097 A	14-04-1998
			CA	2262476 A1	26-03-1998
			EP	0932695 A2	04-08-1999
			JP	2002514054 T	14-05-2002
			WO	9812339 A2	26-03-1998
US 6096534	A	01-08-2000	US	2002110896 A1	15-08-2002
			AT	204329 T	15-09-2001
			AU	695762 B2	20-08-1998
			AU	5924296 A	11-12-1996
			CA	2221708 A1	28-11-1996
			DE	69614527 D1	20-09-2001
			DE	69614527 T2	16-05-2002
			EP	0827547 A1	11-03-1998
			ES	2161362 T3	01-12-2001
			WO	9637625 A1	28-11-1996